CHAPTER 5

Histopathological diagnosis[1]

P. Lépine[2] & P. Atanasiu[3]

In principle, the histopathological diagnosis of rabies consists in recognition of the presence in the animal of acute encephalomyelitis which can be ascribed to a specific agent, namely the rabies virus.

If the animal has died from rabies, it is normally easy to detect the specific lesions; if the animal has been killed, death may have occurred before the appearance of specific lesions (Negri bodies). Consequently, any animal in which the cerebrospinal axis (brain, medulla or ganglia) shows the slightest sign of lesions, particularly infiltrations, should be regarded as suspect, no matter how small the lesions may be.

After correct removal, the brain should be carefully dissected. Smears or impressions should first be examined for Negri bodies (see Chapter 4) or by the FA test or electron microscopy (see Chapter 18). However, while a positive result is indicative of infection, a negative result does not rule out the possibility of infection. A regular histopathological examination should be made of sections stained after embedding by a rapid method. At least six samples should be examined, corresponding respectively to Ammon's horn (both sides), the cerebral cortex (motor area), the cerebellum, the medulla, and a ganglion (gasserian or upper cervical).

The sections should be examined for:

- Signs of meningoencephalomyelitis, i.e. meningitis, meningeal infiltration, perivascular cuffing, parenchymatous infiltration, formation of encephalitic nodules (Babès' tubercles), and ganglion infiltration with satellitosis and neuronophagia (lesions of van Gehuchten and Nélis) (Figs. 5.1 and 5.2). These lesions may be detected by any staining method (e.g. haematoxylin–eosin, polychrome methylene blue). They show the existence of encephalomyelitis and enable a tentative diagnosis of rabies to be reached.
- Specific lesions. The different types of neurons should be examined for Negri bodies and lesions of fixed rabies virus.

The Negri bodies are found especially in the central pyramidal layer of Ammon's horn (Fig. 5.3) and the hippocampus, in the lower loop and the middle layer of the ganglioneurons of Ammon's horn and, less frequently, in the neurons of the cerebellum, the motor area of the cerebral cortex, and the medullary nuclei. They may be present in very large numbers in the ganglia but are generally small in size.

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[1] Based on the chapter in the previous edition, which was prepared by the late P. Lépine and updated by the late P. Atanasiu.

[2] Deceased. Former Director, Virus Department, Pasteur Institute, Paris, France.

[3] Deceased. Former Honorary Professor, Pasteur Institute, Paris, France.

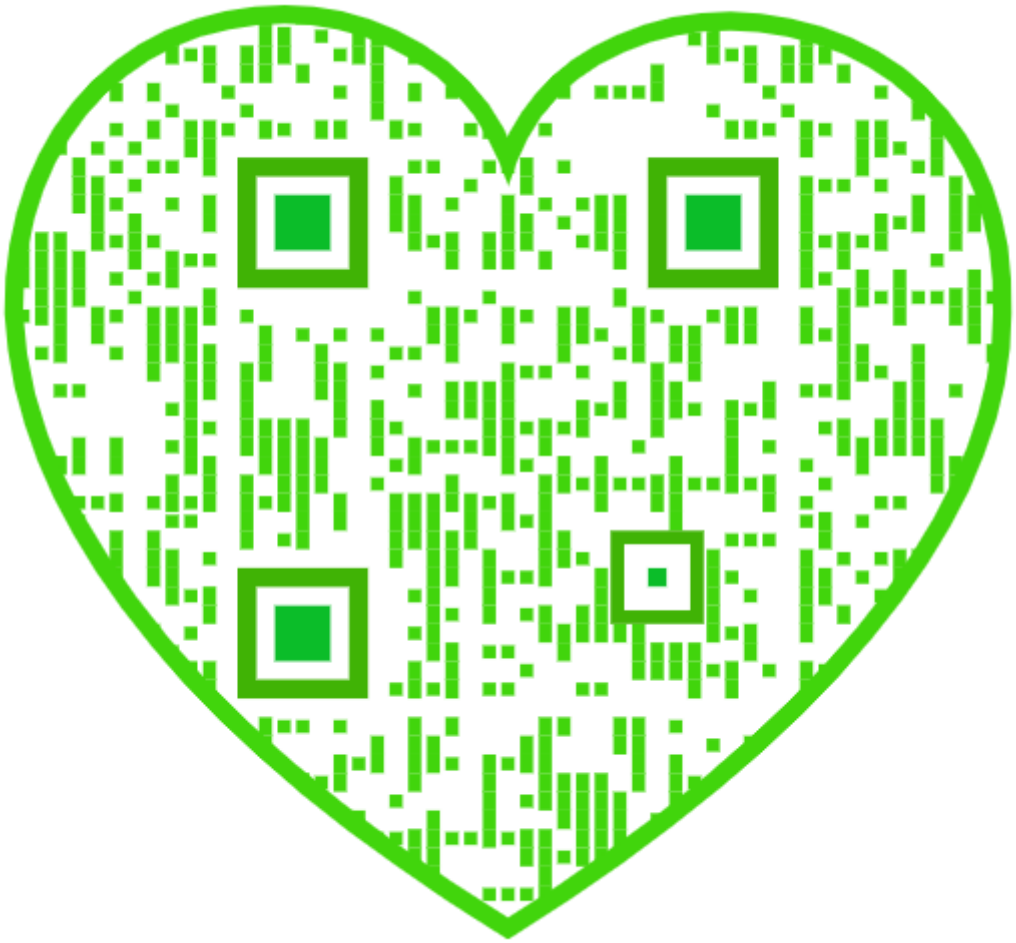


Fig. 5.1 Normal gasserian ganglion

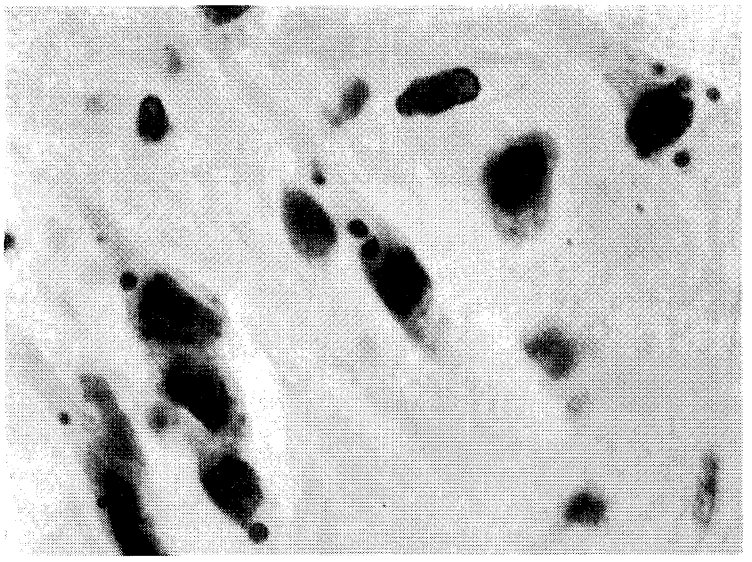
Ganglioneurons with a single layer of satellite cells.

Fig. 5.2 Infiltration of a gasserian ganglion with satellitosis and neuronophagia (lesions of van Gehuchten & Nélis)

The lesions of fixed rabies virus are found exclusively in the middle zone of the external layer of the cells of Ammon's horn. They always coexist in varying proportions with the lesions of street rabies virus.

These lesions can be detected only by special staining methods (Mann, Sellers, etc., see Plate 5.1). Their presence enables a *definite* diagnosis of rabies to be made.

Fig. 5.3 Dog: street rabies virus



(Mann stain: x 2000)
Negri bodies in neurons of the central pyramidal layer of Ammon's horn. (The brain specim was partially decomposed.)

Removal of the brain and preparation of tissue samples for examination[1]

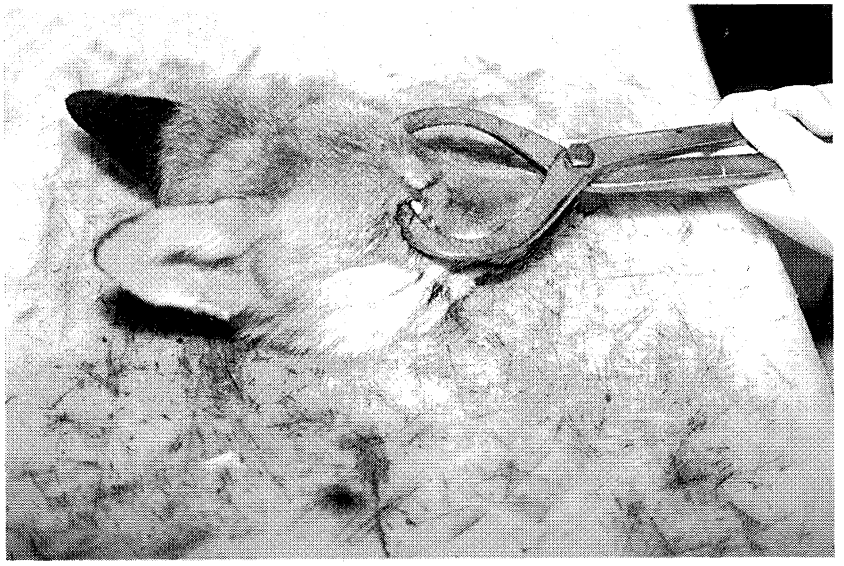
Large animals (dogs, foxes, cattle)

1. Secure the animal firmly to the autopsy table or, better, decapitate the animal and hold the head tightly with forceps (Fig. 5.4). Thick rubber gloves and goggles should be worn to protect the hands and eyes.
2. Make an incision on the midline of the skull through the skin, push aside the flaps of skin, and reflect the muscles and fascia as far as the base of the skull, proceeding from the crown to a horizontal line passing through the eyes (Figs. 5.5 and 5.6).
3. Using a hammer and bone chisel, open the skull at eye level and cut along the temporal bone (Figs. 5.7 and 5.8).

   In the case of very large animals (such as large dogs, cows, etc.) a different method is preferable: make a longitudinal saw-cut on each side of the midline at about 1.5 cm from it, and join up these cuts by one or two transverse saw-cuts above the orbits and at the occiput so that the calvaria can be removed in two symmetrical pieces.
4. Once the calvaria has been removed, use fresh instruments to open the meninges, including serrated dissection forceps and a pair of fine, sterile

[1] See also Appendix 1.

Fig. 5.4 Immobilization of a fox's head with forceps



By courtesy of J. Barrat, Laboratoire d'Etudes sur la Rage et la Pathologie des Animaux sauvages, CNEVA, Malzéville, France.

scissors. Make an incision in the meninges, starting from the median region, along and on each side of the longitudinal sinus.

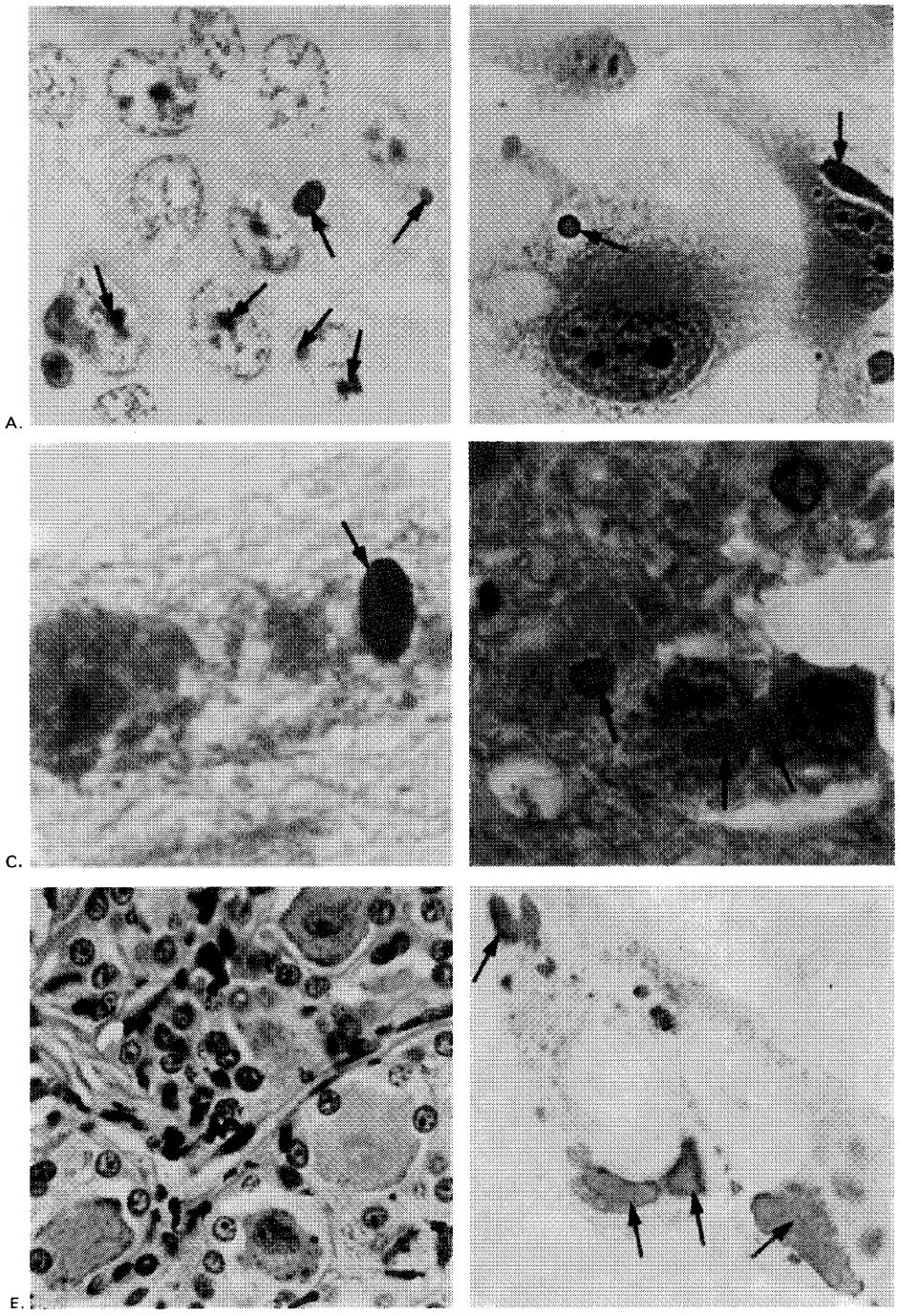
5. Make a second incision perpendicular to the first and push the meningeal flaps upward and backwards.
6. Using fresh instruments, cut through the medulla with a scalpel as low as possible and lift up the brain, proceeding from back to front and successively severing the pairs of cranial nerves.
7. At the end of the operation, roll the brain gently forwards into a large sterile Petri dish so that it rests on its upper surface (Fig. 5.9).
   Note: In hot weather, or if the brain is soft (cadaveric brain), it should be placed in a refrigerator set at 5 °C for 2 hours before dissection.

Examination of the brain

Note whether or not there is congestion of the cerebral vessels or exudate in the meninges, etc. Dissect the brain as follows:

1. Using a brain knife, separate the two hemispheres longitudinally, after having detached the cerebellum and the medulla.
2. Look for the hippocampus or Ammon's horn. This may be done in two ways (see also Chapter 4):

   (a) Cut across the brain transversely, starting from the base behind the optic chiasma and proceeding towards the lower third of the cerebral hemisphere. The third ventricle appears on the cut surface; Ammon's horn is seen as a whitish fold resembling a large bean cut transversely, and can easily be removed.



A.

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E.

(b) Alternatively, a longitudinal incision may be made externally in the posterior third of each cerebral hemisphere about 1.5 cm from the midline. The incision is continued through the grey matter and the white matter until a narrow groove, the third ventricle, is reached. Ammon's horn will be seen on the floor of the ventricle in the form of a glistening white, semi-cylindrical bulge, extending laterally on each side (see Fig. 4.5, page 58).

3. Cut transverse sections 1–2 mm in thickness from each Ammon's horn. Take similar samples from the cerebral cortex (motor area), the cerebellum, and the medulla.

When using the impression method, at least six slides (two for each Ammon's horn, one for the cerebral cortex, and one for the cerebellum) should be carefully examined for Negri bodies before deciding that the results are negative. If the results are negative, however, histological examinations are carried out.

Preparation of the tissue samples for histological examination. If the tissue is soft and difficult to section, prepare pieces of filter paper slightly larger than the tissue sample to be collected. Apply the piece of filter paper to the cut brain surface, grasp the edge of the filter paper with fine forceps held in the left hand, and with the right hand make a cut with a scalpel parallel to the filter paper and 2–3 mm from it, so as to remove the piece of brain. Immediately submerge the tissue sample and the fragment of filter paper to which it is adhering in the fixing agent.

Removal of material for inoculation

During the operation, care should be taken to reserve material from the same areas (cortex, Ammon's horn, cerebellum and medulla) for use in animal inoculation (see Chapter 6). If the brain is received in good condition and can be assumed to be sterile, the samples are removed before any examination is made. When the brain is infected, the samples may be removed at any time and antimicrobials added, as described on page 61.

Plate 5.1

A. Street rabies virus in dog brain. Many typical Negri bodies are situated in the cytoplasm and in the prolongations of the neurons, some of which contain haloes and "Innerkörperchen" (Mann stain: x 1550).

B. Fixed rabies virus in tissue culture. Intracytoplasmic inclusions of different shapes and sizes are observed (Mann stain: x 1550).

C. Street rabies virus in bovine brain. An inclusion in the cytoplasm of a degenerated neuron (to the right of the nucleus) is a typical Negri body with basophilic "Innerkörperchen" (Sellers' stain: x 1550).

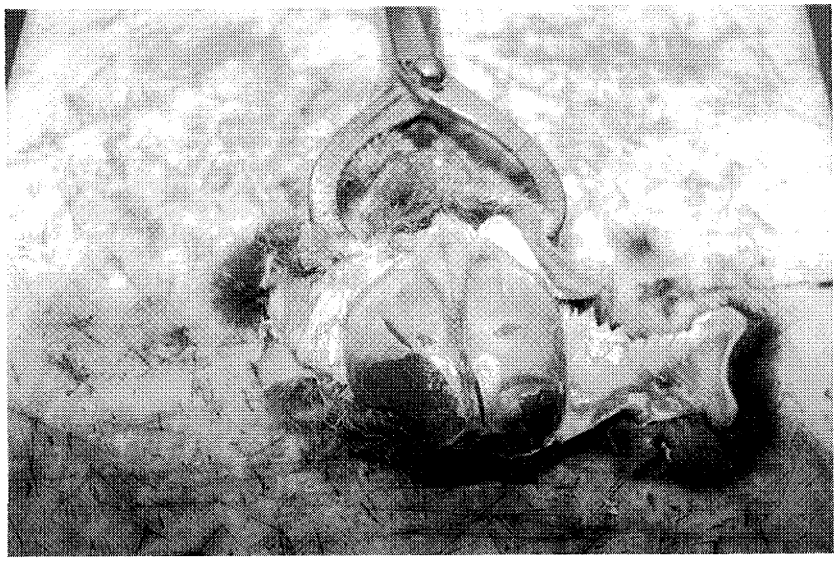
D. Cat brain section with nonspecific inclusions in the cytoplasm. No "Innerkörperchen" (Mann stain: x 1550).

E. Street rabies virus in monkey ganglia. Infiltration and neuronophagia of a gasserian ganglion: chromatolysis and Negri bodies (haematoxylin–eosin: x 660).

F. Fixed rabies virus in tissue culture. Cytoplasmic inclusion (immunoperoxidase: x 1550).

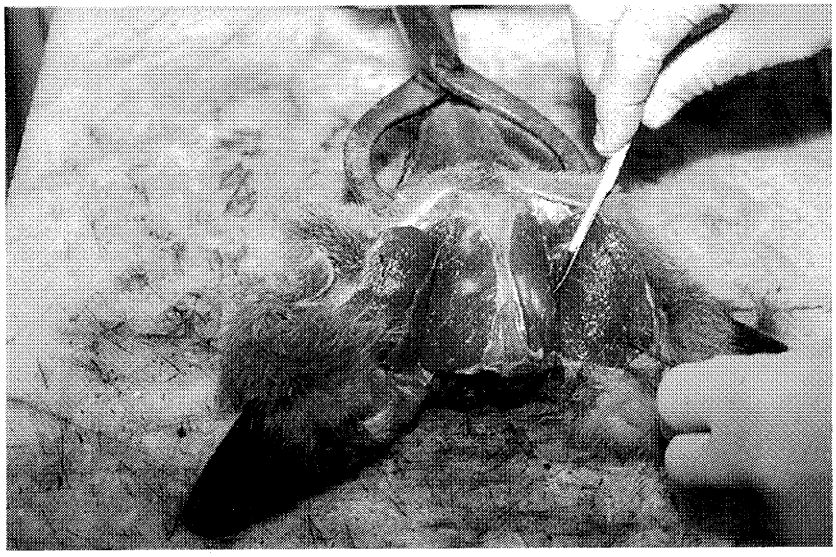
Photographs kindly supplied by the late P. Atanasiu & J. Sisman, Pasteur Institute, Paris, France.

Fig. 5.5 Reflection of the skin, exposing the temporal muscles



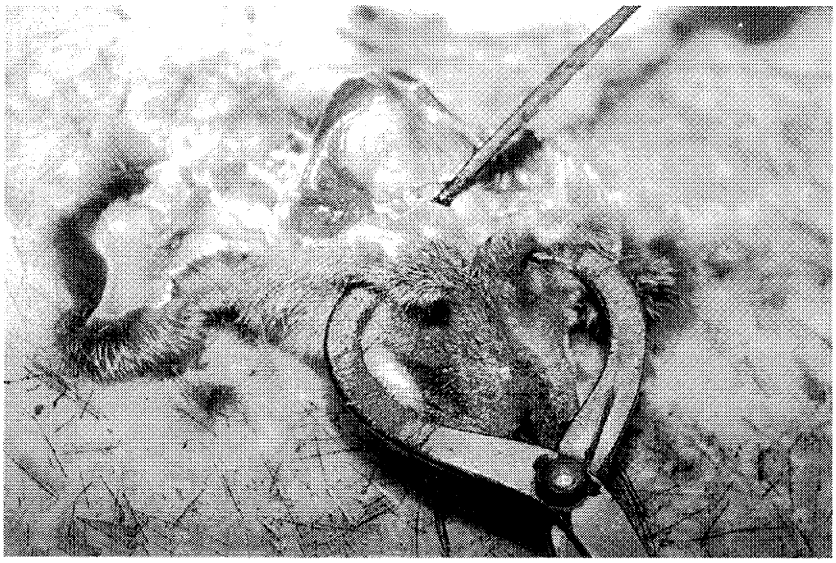
By courtesy of J. Barrat, Laboratoire d'Etudes sur la Rage et la Pathologie des Animaux sauvages, CNEVA, Malzéville, France.

Fig. 5.6 Dissection of the temporal muscles of the cranium



By courtesy of J. Barrat, Laboratoire d'Etudes sur la Rage et la Pathologie des Animaux sauvages, CNEVA, Malzéville, France.

Fig. 5.7 Opening the cranium



By courtesy of J. Barrat, Laboratoire d'Etudes sur la Rage et la Pathologie des Animaux sauvages, CNEVA, Malzéville, France.

Fig. 5.8 Removal of the calvaria, exposing the brain

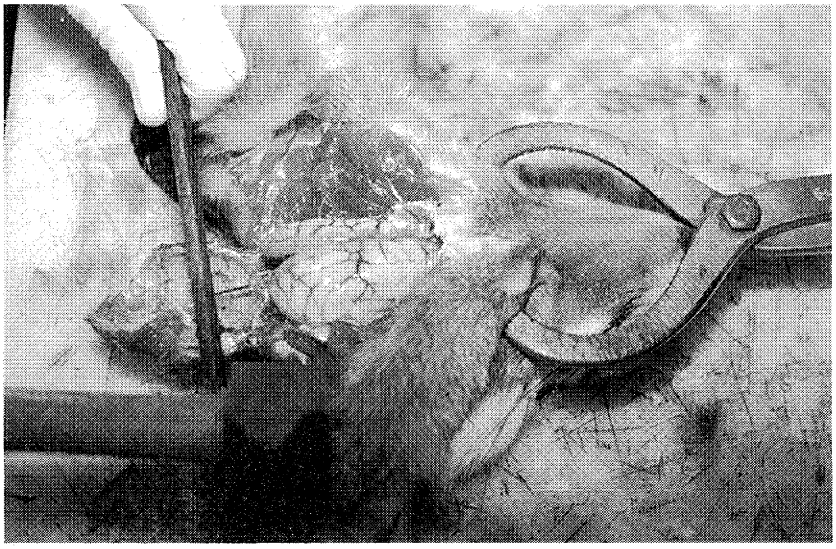


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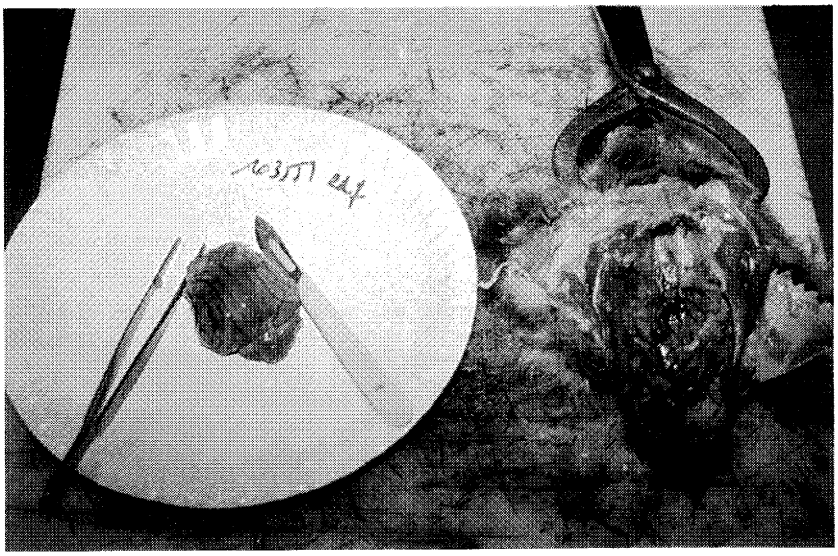
Fig. 5.9 Brain removed from the skull



After the brain has been removed, it should be placed on a plate labelled with the sample no., species and sex of the animal. The scalpel and forceps should be changed after each operation.

By courtesy of J. Barrat, Laboratoire d'Etudes sur la Rage et la Pathologie des Animaux sauvages, CNEVA, Malzéville, France.

Small animals (rabbits, guinea-pigs, hamsters, mice)

Rabbit

1. Place the animal on its stomach and secure it to the autopsy tray, with the head at the edge of the tray.
2. Using a serrated dissection forceps, scalpel and scissors, completely scalp the head from the nape to the muzzle, removing the ears and the upper eyelids.
3. Moisten the exposed surface of the head with 70% ethanol and rapidly flame it with a gas burner.
4. Holding the muzzle of the animal with a Farabeuf forceps in the left hand, open the brain pan with three cuts of the bone forceps. Make the first two in the front part of the head, from each orbit to the midline, opening the brain pan (parietal and temporal bones) in two flaps, to the right and to the left. The third cut is made at the occiput, lifting the bone flap backwards, which completes the clearance of the field of operation. The Farabeuf and bone forceps are then laid aside.
5. Using the fine forceps and scissors, free the meninges and section the anterior part of the brain at the olfactory lobe: after severing the medulla behind the cerebellum, raise the brain in order to cut the optic chiasma and then place it in a sterile Petri dish.

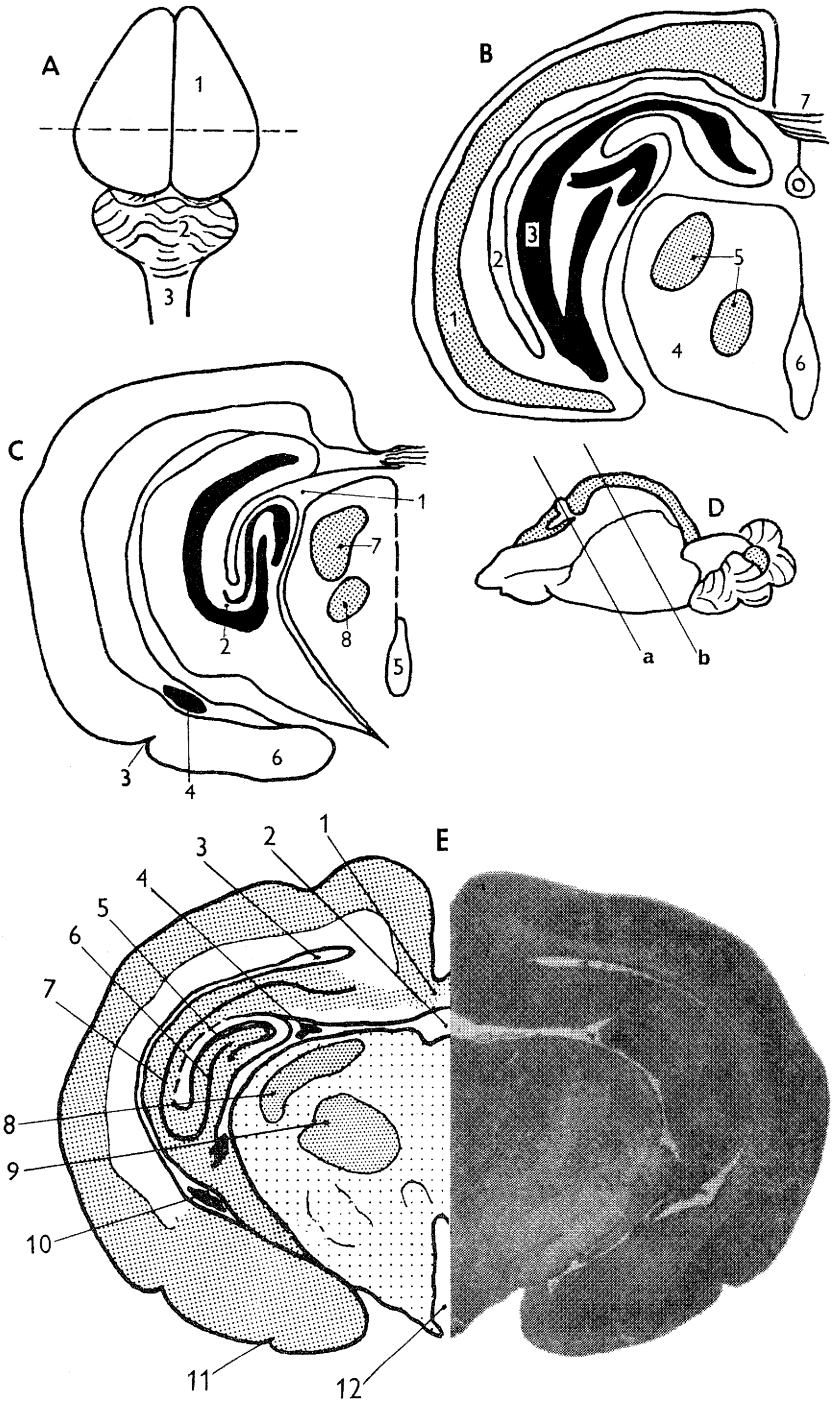
6. With the brain lying on its dorsal surface in the Petri dish, cut the cerebral trunk at the peduncle; then make a transverse cut through the brain substance, starting from the optic chiasma and going towards the convexity, parallel to the posterior surface of the hemispheres and the cut surface of the cerebral trunk. A second cut made in the same way, parallel to the first and 5–8 mm behind it, gives a transverse section of the brain that includes the hippocampal gyrus, Ammon's horn and the basal optic ganglion—areas of choice for the detection of Negri bodies—in addition to the cortical motor area (Fig. 5.10).
7. Cut a transverse section of the cerebellum in order to examine Purkinje's cells and the peduncular region.
8. Finally, cut a slice from the end of the cerebral trunk to obtain a section of the medulla.
9. To reach the gasserian ganglion, cut through the petrosal bone with the bone forceps at its insertion into the sella turcica: force apart the cut surfaces by everting the temporal bone downward and outwards. The gasserian ganglion is easily recognized from its whitish nodular appearance and its almost fibrous consistency.
10. Carefully remove the ganglion, place it on a piece of filter paper, and immerse it in the fixing agent, together with the other samples.

Guinea-pig, hamster

1. Secure the animal to the autopsy tray and scalp the head as described above.
2. Wipe the skull with 70% ethanol and flame it rapidly.
3. Using the second pair of large sterile scissors, open the skull by means of four incisions encircling the skull cavity, the first one joining the two orbits and the other three made successively at the sides and the occipital bone.
4. Remove the brain with the fine forceps and scissors and dissect it as described above for the rabbit. The gasserian ganglion is more difficult to locate than in the rabbit; however, with care, it can be found.

Mouse

1. Place the mouse, ventral surface downwards, on a sheet of cork. Spread the limbs out and secure them with pins. Also secure the base of the tail and the anterior extremity of the muzzle, which should be pulled well forward.
2. Remove the skin from the head.
3. Wipe the skull with 70% ethanol and flame it very gently with the pilot flame of the gas burner.
4. Open the skull with fine scissors, first by joining the orbits, then cutting laterally through the skull fairly low, and finally pushing back the flap thus obtained.
5. Sever the medulla and the optic chiasma.
6. Remove the whole of the brain and place it in a sterile Petri dish.
7. Remove the cerebellum.
8. Cut through the brain transversely at the optic chiasma and along a plane parallel to the posterior surface of the brain, so as to obtain a cross-section; this is placed in the fixing agent, the anterior portion being reserved for smears and inoculations. Also fix a section of the cerebellum.
   If examination of a ganglion is needed, the upper part of the spinal cord should be detached and removed together with the upper cervical ganglion.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Embedding, staining and examination for Negri bodies

Fixation

Bouin–Dubosq–Brazil mixture

When rapid fixation is not necessary, the best fixing agent is Bouin–Dubosq–Brazil mixture. This can be used for all examinations not requiring a special fixing agent or immunohistochemical techniques and is particularly suitable for the staining of nuclear and cytoplasmic inclusions. The formula is as follows:

Formaldehyde, 40% solution	500 ml
Ethanol, 96% solution	1100 ml
Distilled water	100 ml
Glacial acetic acid	120 ml
Picric acid	8 g.

Prepare the tissue sections and fix for 24 hours in this mixture. When fixation is complete, dehydrate the tissue in 96% ethanol and then in absolute ethanol. Finally, embed the specimens in paraffin, using the following technique.

Embedding techniques

Rapid method for histological embedding

For slices of brain tissue 1 mm in thickness, fixation is complete in 15–30 minutes if a rapid fixation method is employed. The tissue is transferred directly to absolute ethanol, with which it is treated for 20–30 minutes in two baths, followed by two changes of toluene and two of paraffin, each lasting 15 minutes. Including the time necessary for cutting and staining the sections, the preparation is ready for microscopic examination about 3.5–4 hours after autopsy of the animal.

Staining techniques

Sellers' method

Sellers' method may be used for staining sections prepared using standard techniques. Thus, the simplicity of Sellers' method of staining is combined with the

Fig. 5.10 Sections of rabbit and mouse brain

A. Mouse or rabbit brain: (1) cerebrum; (2) cerebellum; (3) medulla oblongata.
B. Mouse brain: (1) cerebral cortex; (2) lateral ventricle (choroid plexus); (3) Ammon's horn; (4) mesencephalon; (5) inner nuclei of grey matter; (6) median ventricle; (7) corpus callosum.
C. Rabbit brain: (1) third ventricle; (2) Ammon's horn (middle layer); (3) rhinal fissure; (4) choroid plexus; (5) infundibulum; (6) hippocampal gyrus; (7) lateral geniculate ganglion; (8) ventral nucleus of thalamus.
D. Rabbit brain after removal from cranial cavity (inverted, lateral aspect): left—anterior portion; centre—temporal and parietal lobes; right—cerebellum. Section is performed anterior to a (through optic chiasma) and posterior to b; the portion a–b is immersed in fixative.
E. Rabbit brain, transverse section a–b: (1) corpus callosum; (2) third ventricle; (3) lateral ventricle; (4) choroid plexus; (5) Ammon's horn (middle layer); (6) Ammon's horn (inner layer); (7) Ammon's horn (outer layer); (8) lateral geniculate ganglion; (9) ventral nucleus of thalamus; (10) lateral choroid plexus; (11) rhinal fissure; (12) infundibulum.

By courtesy of the late P. Atanasiu.

greater accuracy afforded by histological examination, so that the Negri bodies can be observed *in situ* in the cells, and all possibility of error is excluded.

The preparation of Sellers' stain is described on page 62. After the paraffin is removed, the sections are stained by immersion in a mixture of 6 ml of basic fuchsin stock solution, 20 ml of methylene blue stock solution and 50 ml of absolute methanol.

The time required for staining depends on the thickness of the section; it is usually between 2 and 10 minutes. The stained sections are washed in tap-water, dried with filter paper without washing with alcohol, and mounted in balsam.

Results: Negri bodies, deep magenta red; neurons, blue-violet; inner bodies of the Negri bodies, dark blue to black; nucleoli of the neurons, dark blue; erythrocytes, copper red (see Plate 5.1,C and Chapter 4).

Mann's method

This classical method gives sections that are permanently stained, with very fine differentiation of the Negri bodies. It is an excellent method for demonstrating the presence of Negri bodies, but it requires time and a certain dexterity for full success.

Prepare the following mixture immediately before use:

Methyl blue (*not* methylene blue), 1% aqueous solution	18 ml
Eosin, 1% aqueous solution	23 ml
Distilled water	49 ml.

Stain the sections for 24 hours at room temperature, or for 6–14 hours at 38 °C (in this case first treat the sections with a 1:1 mixture of absolute methanol and 10% formaldehyde in distilled water to render the gelatine insoluble and prevent the sections from becoming detached).

Wash with tap-water and then rapidly with absolute ethanol.

For differentiation, use the following solution:

Sodium hydroxide in ethanol, 1.5% solution	1 ml
Absolute ethanol	30 ml.

Leave the section in this solution until it is stained pink (about 10 minutes). As soon as this stage is reached, wash the preparation well with tap-water. The section should take on a sky-blue colour; if not, treat it with water containing acetic acid (2 drops of acetic acid in 40 ml of distilled water) for 1 minute.

Dehydrate rapidly (absolute ethanol), treat with xylene, and mount in balsam.

Results: Negri bodies, vermilion red; nucleoli of the neurons, violet-red; chromatin, blue; cells, dark blue; stroma, pale blue; erythrocytes, pink (see Plate 5.1, A, B, page 70).

If phloxin B is substituted for eosin in the same proportion, the preparations obtained are less attractive (purplish-blue or mauve background instead of sky-blue), but the inclusions (Negri bodies) are more numerous and more striking.

Fuchsin-safranine-blue method

After fixation, the tissue is embedded in paraffin, cut into thin sections, and freed from the paraffin.

1. Stain for 10 minutes with the following mixture:

   Solution 1: Basic fuchsin 1 g
   Ethanol, 50% solution 200 ml
   Solution 2: Safranine, 0.2% aqueous solution.

   Mix equal parts of the two solutions and store in a dropping bottle; the mixture is fairly stable and keeps for some time.
2. Discard the stain, cover the section with a mixture of ethanol and acetone (equal parts) to remove excess stain, and wash rapidly: the section is coloured red.
3. Stain for 15–60 seconds with Unna's polychrome methylene blue (10% dilution) or with permanganate blue prepared by Stévenel's method and used undiluted.
4. Discard the stain: the section is deep violet in colour.
5. Differentiate in ethanol–acetone for a few seconds: the section becomes blue.
6. Wash the preparation immediately in running tap-water to remove excess stain, and again treat with ethanol–acetone.
7. Without washing the preparation, partially dehydrate it by shaking it in a Borrel tube filled with absolute ethanol. The remaining stain is removed from the section which becomes differentiated, taking on a pink-lilac tint varying in paleness according to the thickness of the section.
8. Rapidly complete dehydration in absolute ethanol, carefully remove the alcohol in several changes of xylene or toluene, and mount in balsam.

Results: Stroma, very pale pink with nerve fibres a deeper pink; neuroglia and leukocytes, purplish-blue; neurons, light blue; chromatin, deep purple with the nucleolus a vivid red; nuclear inclusions and oxyphilic substances, bright pink; Negri bodies, poppy-red to mauve pink, with the internal structure lilac.

CHAPTER 6

The mouse inoculation test

H. Koprowski[1]

The mouse inoculation test, in spite of its simplicity, depends greatly on the accuracy of its performance for dependable results.

Choice of mice

Strain

White mice of any breeding strain may be considered suitable, although preference should be given to the Swiss albino strain since it is very susceptible to rabies virus and it is easy to maintain the breeding stock in the laboratory. If the Swiss albino stock is not available, however, almost any breed of mice, except grey wild mice, can be used, because a genetically resistant strain has not yet been found. Grey wild mice should be excluded because of the difficulty in restraining these animals in cages during the observation period.

Age

Suckling mice (preferably less than 3 days old) are more susceptible to intracerebrally inoculated rabies than weanling or adult mice and should be used wherever possible. The use of suckling mice has permitted individual virus isolations that would have been missed if only young adults had been inoculated. If sufficient mice are available, the mouse inoculation test should be performed routinely, in addition to other diagnostic tests. Since confusion may arise from nonspecific deaths, the combined use of the mouse inoculation test and the FA test is recommended.

Sex

Mice of both sexes are equally susceptible to rabies virus. It is inadvisable to keep older mice of the same sex in one cage since they are apt to kill each other in fights before the observation period is completed. This applies especially to males.

General health

It is imperative that the animals chosen for inoculation be in good health. It is important to know the history of the breeding colony, and it is advisable to inspect the animals closely before inoculation.

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[1] Director, Center for Neurovirology, Jefferson Cancer Institute, Thomas Jefferson University, PA, USA.

Any animals found to have ectoparasites, ruffled fur or signs of diarrhoea should be withdrawn from the test immediately. If the mice are sent to the laboratory from a considerable distance, it is advisable to postpone inoculation for at least 3 days in order to let them rest and become adjusted to changed conditions. In such cases, it may be equally important to leave a few animals from the shipment uninoculated in order to observe the death rate among "normal" mice as compared with inoculated animals.

Preparation of suspect material for inoculation

Choice of tissue

Either the brain or the salivary-gland tissue of a suspect animal may be used for virus isolation. Detection of the virus is more frequently possible in the brain than in the salivary glands. However, from the epidemiological and epizootiological points of view, it is important to examine the salivary glands for the presence of virus.

Although it is relatively immaterial which part of the brain tissue is chosen for the preparation of the suspension, preference is usually given to Ammon's horn, the cerebellum and the cerebral cortex. When salivary-gland tissue is used, the submaxillary glands are those most likely to show the presence of rabies virus. In addition, it is always advisable to mince salivary-gland tissue before grinding.

Grinder

The choice of grinder depends to a certain degree on the amount of tissue available. If more than 3–4 g of material are available, a small Waring-type blender should preferably be used. If the amount of tissue is less than 3 g (which is more usually the case), or if a Waring-type blender is not available, the following grinders or grinding devices may be substituted, in the order of preference listed.

(a) *TenBroeck grinder:* This grinder is easy to assemble, manipulate, clean and sterilize, but only brain tissue may be used in it since salivary-gland tissue is too tough for it to grind properly. A slight disadvantage is its fragility. If it is not used properly, it may break while being manipulated and it may also be damaged during cleaning, sterilizing, etc. This grinder and the Waring-type blender, if properly used, have advantages over the other grinding devices.

(b) *Pestle and mortar:* This is a time-honoured method of grinding and it has the advantage that, with the help of an abrasive such as sterile sand, even the toughest tissue can be properly ground up. The pestle and mortar cannot be operated under such sterile conditions as can the Waring-type blender or the TenBroeck grinder; however, it can be easily cleaned and sterilized, and stands wear and tear for a long period of time. It is also cheaper than the other grinding devices.

Diluent

The choice of diluent may be left to the user, but an isotonic salt solution is preferable, although mice have been known to withstand easily an intracerebral inoculation of sterile distilled water. The following diluents may be considered in order of their availability:

(a) *A physiological salt solution containing varying amounts of animal serum (10–50% concentration):* This is by far the commonest diluent used. It should be carefully ascertained, however, that the donor animal has never been vaccinated against rabies. It is therefore advisable to avoid the use of dog, cat or cattle serum. Normal sheep serum seems to possess some "antiviral" properties that are absent from rabbit serum. The rabbit should therefore be considered the preferred source of blood. The serum should be inactivated by heating it for 30 minutes at 56 °C before using it as a component of the diluent. Diluent-containing serum should be sterilized by filtration through bacteria-retaining filters.

(b) *Other diluents:*

— skimmed milk;
— 2% bovine serum albumin in buffered salt solution;
— physiological salt solution in distilled water.

These are not particularly recommended, although rabies virus seems to be relatively non-susceptible to the inactivating action of salt solution, in contrast to such viruses as eastern or western equine encephalomyelitis.

Note: If the tissue suspension is to be frozen and stored, a 50% solution of serum in distilled water should be the preferred diluent.

Bacterial sterility

There is no need to add antimicrobials to suspensions of *brain tissue* if the material has been handled with reasonable precautions at the autopsy and dispatched in a sterile container. A 50% glycerol solution is highly recommended for preserving the material since, in addition to its preserving qualities, it exerts a strong bacteriostatic action (see Chapter 4, page 62). If bacterial contamination is suspected, however, streptomycin and penicillin should be added to the suspension to give a final concentration of 1560 IU of streptomycin and 500 IU of penicillin per ml. If antimicrobials are added, the suspension should be left to stand for at least 30 minutes before the animals are inoculated.

It is always advisable to add antimicrobials to suspensions of *salivary glands.* The salivary-gland tissue suspension should also be cultured for possible bacterial contamination. Beef-infusion broth, thioglycolate medium, and blood-agar are considered to be good culture media for this purpose. If bacterial growth is observed, an attempt should be made to identify the bacterial agent. If the results of the mouse inoculation test are equivocal (see page 86), it may be advisable to test the pathogenicity for mice of the bacterial contaminant by intracerebral inoculation.

Concentration of infected tissue in the suspension

This is optional. If the suspension is for storage, prepare a 20% suspension by weight. The weight of the tissue in grams multiplied by 4 gives the required volume of diluent in millilitres. However, if the suspension is to be used for inoculating mice intracerebrally, a 10% suspension by weight should be prepared. Either dilute the

20% suspension by adding an equal volume of diluent, or prepare the suspension by multiplying the weight of tissue in grams by 9 in order to obtain the required volume of diluent in millilitres.

In attempting to isolate certain strains of rabies street virus, interference phenomena may be suspected (see page 86). In such cases it is advisable to dilute the tissue suspension more than 10%.

Centrifugation and filtration

If the equipment is available, centrifuge the tissue suspension for 5 minutes at 150–200 *g* to remove the gross particles. However, if no centrifuge is available, mice may be inoculated intracerebrally with a 10% uncentrifuged brain suspension. Salivary-gland suspensions, if uncentrifuged, must be filtered through one or two layers of sterile gauze in order to prevent the animals dying from trauma.

Inoculation of mice

Choice of syringe

Syringes that can measure accurately 0.03 ml (a single mouse dose) should be used. Thus 0.25-ml tuberculin syringes should be considered first, followed by 0.5-ml or 1-ml tuberculin syringes. For intracerebral inoculation, the needles should be 0.40–0.45 mm in diameter (27- or 26-gauge) and 1.0–1.5 cm long. Larger needles cause trauma to the brain substance.

Anaesthesia

The mice should be anaesthetized before they are inoculated, preferably using ether. A battery jar with a specially fitted wire bottom may be used for this purpose. If no such device is available, pentobarbital sodium injection should be considered. Ideally, the table used for mouse inoculation should be placed well away from the wall, so that an assistant can etherize the mice from a position opposite the operator.

Inoculation technique

There is a wide choice of methods of inoculation. For a right-handed operator the following technique has been found satisfactory.

1. Place the anaesthetized mouse on its left side, with the legs pointing towards you.
2. Support the lower jaw of the animal with the left thumb, and place the left index finger behind the skull of the animal. Very little pressure should be exerted, otherwise the animal may be asphyxiated and die. Hold the syringe in your right hand in a horizontal position parallel to the table surface and perpendicular to the head of the mouse, with the needle pointing towards you. With a quick thrust, push the needle through the skull of the animal at the place that can best be described as the apex of an imaginary angle, the arms of which point to the animal's right eye and right ear. The needle should easily penetrate the bone

and should then be further inserted for about 0.1–0.2 cm into the brain tissue. If a 1.5-cm needle is used, care should be taken not to penetrate too far, otherwise the injection may be given into the base of the skull. Push the plunger to the next 0.03-ml mark and then gently withdraw the needle. Move the inoculated mice away from the syringe hand (i.e. right-handed persons pass the inoculated mice to the left). This is to prevent crossing hands, which may result in catching a finger on the needle held in the opposite hand.

3. Place the inoculated mice immediately in a can or box previously prepared and identified by a tag bearing the mouse-group number or any other particular identification mark. If several groups of mice are inoculated, it is advisable to check the number of living mice in each group after the inoculations are finished. If any animals are found dead, an equal number of new animals should be inoculated and added.

Under no conditions should the same syringe be used for inoculation of two different suspensions. If an adequate number of sterile syringes is not available, each syringe should be boiled between inoculations, and care should be taken to let it cool before filling it with inoculum.

Strict safety precautions should always be followed whenever the rabies virus is handled (see Chapter 1). For example, the rapid emptying of the syringe used for inoculation into a pan of water will produce an aerosol that can cause infection in the operator, or in the animals with which he or she is working. Virus may be spread from the table used for animal inoculations to the hands, and if these are not washed properly it is possible to contaminate subsequent specimens during grinding of tissue in a mortar — for example, virus may be transferred from the hands or sleeves into the mortar. Mice laid on the table after etherization may awake and have to be put back in the same ether jar, thus contaminating it. Should other studies be carried out using the same ether jar, virus may be deposited on the head of normal mice and be carried into the brain by the inoculation procedure. The table used for the inoculations should therefore be considered contaminated until it has been washed with soap and water and treated with an appropriate disinfectant (see Chapter 1).

When inoculation of all the mice is finished, the syringe and needle may be rinsed in water, provided this is done gently with the point of the needle well below the surface of the water in the sterilization tray. The inoculation of mice and harvesting of mouse tissues is best done on trays that can be sterilized and covered with absorbent paper backed with polyethylene or aluminium foil. Instruments used for individual specimens should be sterilized by autoclaving (121 °C for at least 20 minutes), dry heat (170 °C for 2 hours) or by continuous boiling (for 20–30 minutes); sterilization by flaming between taking specimens may not kill the virus.

Observation of inoculated mice

Although rabies virus will only rarely cause signs of illness in mice before the fifth day after intracerebral inoculation, it is advisable to check mice daily, beginning with the first post-inoculation day. The number of mice found normal, sick or dead is recorded on a mouse-history card which remains on file as a permanent record of the experiment. The observation period should extend for at least 21 days

after inoculation. Only rarely will rabies virus be detected in the inoculum later than 21 days after the inoculation.

The following signs should be noted on the mouse-history card, using the appropriate letter(s):

A. Ruffled fur.
B. Tremors when held in the air by the tail with a pair of forceps.
C. Lack of coordination of hind legs — note gait when placed on table and made to move.
D. Paralysis.
E. Prostration (near death).

Deaths occurring 24–48 hours after intracerebral inoculation are attributable to causes other than rabies virus, such as trauma, bacterial contamination or other viruses. For diagnostic purposes, 1 or 2 mice may be killed each day, beginning on the fifth day, and their tissues examined for Negri bodies as well as for the presence of rabies antigen as demonstrated by immunofluorescent antibody staining. Frequently, an early diagnosis is thus obtained, particularly in instances where certain strains of street virus might take between 1 and 3 weeks to kill the mice.

Note: Rabies should not be diagnosed on the basis of clinical signs of the disease alone. Although signs of paralysis 5 days or more after inoculation may give grounds for suspecting the presence of rabies virus, the same signs may be observed in numerous other viral, bacterial and protozoal infections that involve the central nervous system of the mouse. Definite evidence of the identity of the virus is obtained with the mouse neutralization test.

Further passages of infected material

If desired, brain tissue from mice that have succumbed to infection after inoculation with the original virus may be made into a suspension as described above. It can then be stored, used in neutralization tests, or inoculated into another group of mice.

Removal of the brain

The brains of all mice that have died during the experiment, or that have been killed when prostrate, should be removed and examined for the presence of Negri bodies and rabies antigen by immunofluorescent antibody staining (see Chapters 4 and 7). The mice showing symptoms of prostration (terminal symptoms of rabies) should be killed with chloroform.

The mouse should be pinned, ventral surface downwards, to a dissecting board. Only two pins are necessary, one through the nose and one through the base of the tail. Three pins or spring clips may also be used: one through each of the forelegs, and one through the back of the tail.

After disinfection with 70% ethanol, the skin of the head and neck is cut away with forceps and scissors, exposing the skull. The skull is grasped in the orbits with mouse-tooth forceps and the calvaria of the skull is cut away with curved scissors, thereby exposing the brain. The brain is removed with curved scissors and is

transferred to a sterile Petri dish. A thin section of the brain is cut out, just anterior to the cerebellum, and is transferred to a wooden tongue-depressor or a paper towel. A clean microscope slide is then pressed lightly against the cut surface of the section; the pressure should be sufficient to create a slight spread of the exposed surface against the slide. Negri-body stain and immunofluorescent antibody stain should then be applied to the slide (see plate 5.1, B and C, page 70).

Complications

Bacterial contamination of inoculum

If bacterial contaminants have caused the death of the mice in spite of the addition of antimicrobials, and if the original suspension has been preserved, the following methods may be tried in an effort to overcome the interfering action of the bacteria.

(a) *Filtration through bacteria-retaining filters:* The supernatant liquid of a suspension centrifuged at 400 *g* for 15 minutes should be used for this purpose. However, since rabies virus is a fairly large particle and the concentration of virus in specimens submitted from the field is not usually very great, the virus may be lost in the process of filtration.

(b) *Dilution method:* Sometimes the suspension can be diluted beyond the end-point of bacterial contamination with retention of viral activity, but this happens very rarely.

(c) *Prolonged storage:* In some instances, it is easier to overcome the effects of bacterial contaminants after the tissue suspension has been stored for a period of time either at freezing temperatures or in glycerol (see pages 62 and 82).

(d) *Parenteral inoculation:* The Syrian hamster, which is the animal most susceptible to parenteral infection, may be chosen for this purpose. Mice are relatively insusceptible to parenteral infection with rabies.

Presence of two viruses

This is particularly confusing if the second virus has pathogenic properties similar to those of rabies. Again, intracerebral or parenteral inoculation of animal species other than the mouse may be attempted, particularly in view of the extremely wide host-range of rabies virus.

Interference phenomena

In certain instances, it may be necessary to dilute the inoculum 10–100 times, or even more. This may be because of the properties of a particular strain of rabies virus or because large amounts of inactive virus particles, serum antibody, or other unidentified factors may interfere with the living virus. There is no rule for determining when this should be done. However, if failure to isolate rabies virus is consistently encountered in the same species of animal in a particular geographical area, the possibility of an interference phenomenon should be considered, and tissue suspensions should be diluted beyond 10% concentrations.

Mouse-history card

No.					Date										Virus						
Strain					Passage										Preparation						
Volume					Dilution																
Mouse no.	No. of days post-inoculation																				
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1																					
2																					
3																					
4																					
5																					
6																					
Checked by																					
Mean age of mice					Route of inoculation																

CHAPTER 7

The fluorescent antibody test[1]

D. J. Dean,[2] M. K. Abelseth[3] & P. Atanasiu[4]

The fluorescent antibody (FA) test is one of the most accurate microscopic tests available for the diagnosis of rabies and should be employed by all laboratories undertaking such work. Apart from a satisfactory microscope, the main requirements for success in using this technique are well trained staff and conjugated serum of good quality. When properly performed, the test is fast, comparatively inexpensive, and more accurate than either the examination of films or sections by recommended procedures or mouse inoculation tests. Fresh, frozen or glycerolated material may be examined. Diagnosis can be made accurately in most instances in minutes or hours, whereas the examination of sections or animal inoculation requires days or weeks.

Principle

The fluorescent antibody procedure was developed by Coons & Kaplan in 1950 and subsequently modified for the diagnosis of rabies by Goldwasser & Kissling in 1958. The procedure consists in labelling antibody with a fluorochrome, allowing the labelled antibody to react with specific antigen if present, and observing the product of the reaction under the fluorescence microscope. A substance is said to fluoresce if, upon absorbing light energy at a certain wavelength, it emits light of another wavelength.

Antigens reacting with antibodies tagged with fluorescein isothiocyanate, the dye most frequently used with rabies, appear under ultraviolet light as brightly coloured, apple-green or greenish-yellow objects against a dark background, which may or may not contain nonspecific fluorescing material. The character and intensity of the colour may be modified by the use of filters. Fluorescein isothiocyanate is available commercially in powdered form and may be stored for long periods without deterioration.

The fluorescence microscope consists of a standard microscope of satisfactory quality equipped with a darkfield condenser and an ultraviolet light source. Since only a small portion of the incident radiant energy is converted to fluorescent light, examinations should be made in a darkened room employing the most intense source of light available; both excitation and barrier filters are used.

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[1] Based on the chapter in the previous edition, which was prepared by D. J. Dean & M. K. Abelseth and updated by the late P. Atanasiu.

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Although a monocular microscope will yield more light, a good quality binocular microscope will provide sufficient light and is preferred in diagnostic laboratories where a large number of specimens are being processed. Light used for excitation is of a shorter wavelength than that emitted by the preparation. Blues and greens are excited only by ultraviolet light, while yellow and red fluorescence may also be excited by blue-violet light.

Several manufacturers provide complete kits for fluorescence microscopy and equipment is available for converting standard microscopes of satisfactory quality for use in fluorescence work. Guidance in the selection of a microscope should be obtained from those experienced in fluorescence microscopy and the manufacturer should be required to demonstrate its capabilities prior to purchase. Apochromatic or fluorite objectives allow higher apertures and therefore present brighter images. Achromatic objectives can be used but provide less brilliance. The use of a dry sub-stage darkfield condenser should be considered since it eliminates the use of oil between the slide and front lens of the regular condenser. Large amounts of oil on the microscope may be a hazard since the methods described to process rabies specimens do not inactivate the virus.

An epifluorescence microscope with a ×40 objective is recommended. The mercury or halogen lamp should be checked regularly and changed if necessary.

Materials and methods

Conjugate

Once suitable microscopic equipment has been obtained, the quality of fluorescence is primarily dependent upon the quality of the conjugate used. Some laboratories use sera from hamsters immunized according to schedules similar to those developed by the California State Department of Health in the USA. Other laboratories use equine or goat antisera for conjugation. Since conjugates of acceptable quality are now available commercially, only laboratories familiar with such procedures should attempt to make their own. Such conjugates should be checked and calibrated against reference sera before they are used for diagnosis. Conjugates prepared in the laboratory should be lyophilized to prevent loss of potency. Good quality conjugates should have a minimum of nonspecific fluorescence at low dilutions and should be capable of being diluted as much as 1:20 or more to prevent this problem. After a conjugate lot has been prepared, dilutions should be made and tested to determine which one is most satisfactory for routine diagnosis.

As needed, lyophilized conjugate is removed from the deep freeze and reconstituted to its original volume with sterile distilled water. It should then be filtered to remove extraneous materials. The desired amount of reconstituted conjugate is added to the appropriate volume of normal or infected mouse-brain suspension prepared with egg yolk as described on page 90. For example, if satisfactory staining is obtained with a final dilution of conjugate of 1:40, 0.1 ml of conjugate is added to 3.9 ml of mouse-brain suspension. The remaining undiluted conjugate is stored at 4 °C until use. After storage, the conjugate may need to be centrifuged at 200 *g* for 5 minutes to remove precipitate before it is added to the mouse-brain suspension. Diluted conjugate may be used for a period of a week or so, provided precautions are taken to safeguard against undetected decay of the

product or contamination. If the conjugate is prepared as outlined in Appendix 2, the final dilution may be made with sterile distilled water rather than with the normal mouse-brain suspension as described. This is possible since very little nonspecific fluorescence is found in conjugates prepared in this manner. It should be noted that conjugate prepared in the laboratory may contain nonrabies fluorescein-labelled antibodies if multiple antigens are present in the antigen used in the preparation of antisera. Such conjugates may react when autogenous tissues (from different animals of the same species) are being examined. Care must be taken therefore to select pure rabies antigen.

An antinucleocapsid conjugate has recently been prepared in the rabbit. This conjugate has no nonspecific fluorescence.

Preparation of slides

Two methods of preparing slides are commonly used, the smear method and the impression method (see Chapter 4). Uniform, thin films can be prepared by grinding hippocampal or other brain material without alundum to a uniform paste with a sterile pestle and mortar or by cutting this material into small pieces with scissors. Films provide a greater number of cells for examination. This method is preferred for preparing brain material that has partially decomposed. Impression smears of such material would be too thick for successful examination.

Control slides

Control films or smears are usually prepared from the entire brains of mice or young hamsters inoculated intracerebrally with rabies street virus and killed when moribund. After fixation in acetone, slides can be stored under dry conditions at −20 °C for long periods before use; however, slides no older than 10 days are preferred.

Mouse-brain suspension

When infected mouse brain is used as antigen for preparing antisera, it is usually necessary to dilute conjugates with a 20% normal mouse-brain suspension in PBS, pH 7.4 (see Chapter 8, Annex 1), supplemented with 10% suspension of the yolk of chicken eggs embryonated for 6–7 days. After centrifugation for 10 minutes at 1000 *g*, the supernatant is dispensed in glass containers in aliquots sufficient for 1–7 days' work and stored at − 20 °C until used. Suspensions of infected mouse brain are similarly prepared using brains from young adult mice inoculated intracerebrally with a 1:100 to 1:1000 suspension of the CVS strain of fixed rabies virus and harvested when moribund.

Immunofluorescent staining

Many variations have been developed in the procedure for immunofluorescent staining; however, the direct method is the most satisfactory and practical for rabies diagnosis and will be the only one described.

The direct test

The direct method consists in applying a suspension of rabies antibody, previously labelled with fluorescein isothiocyanate, directly to films, impression smears or sections of tissue under examination (Fig. 7.1).

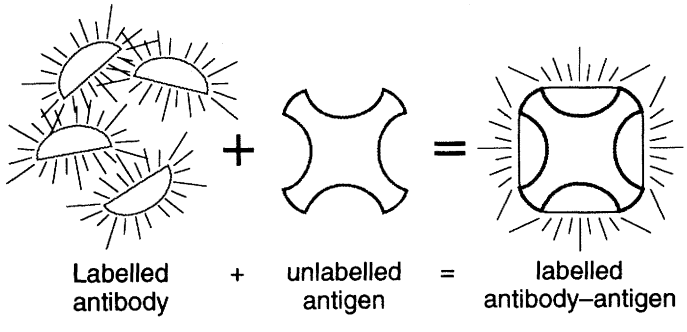
No less than four films of salivary-gland tissue and/or brain tissue should be examined before a field specimen is reported as being fluorescent-negative. They should include two made from tissue from Ammon's horn and two from a paste prepared by grinding in a mortar, without diluent or alundum, equal portions of tissue from Ammon's horn, the cerebellum and medulla oblongata (brain stem). Each of these parts may be examined separately if desired. In examining bovine specimens, the cerebellum and brain stem are tissues of choice. When the mouse inoculation test is used, the remaining ground tissue is diluted with distilled water containing 5% normal horse serum to obtain a 10% tissue suspension and then injected intracerebrally. A direct comparison of the results of fluorescence and mouse inoculation is thus obtained.

When specimens are received in 50% glycerol–saline (preservative), it is imperative that the tissue be washed several times in saline. Glycerol may interfere with the FA test because acetone combines with glycerol to mask fluorescence. In such cases satisfactory results may be obtained by omitting the acetone fixation step.

Films are air-dried, placed in a Coplin jar or other suitable container, covered with cold acetone, and held in a freezer at $-15\,^{\circ}C$ to $-20\,^{\circ}C$ for 2–4 hours; excellent results are also obtained with overnight fixation. After fixation, slides are removed from the acetone and air-dried. If films are used, two suitable uniformly thin areas 2.5 cm long are demarcated on the slide with a wax marking pencil. Impression smears, two per slide, are similarly demarcated. Slides, including previously prepared controls, are then placed on glass rods strategically arranged across the top of a small sterilizing tray or other suitable container.

One area of film or one impression smear is stained by placing two drops of the diluted conjugate within the area marked by the wax pencil. The other film or smear is stained in the same way with conjugate similarly diluted with infected mouse-brain suspension. Conjugate should be spread uniformly, without disturbing the

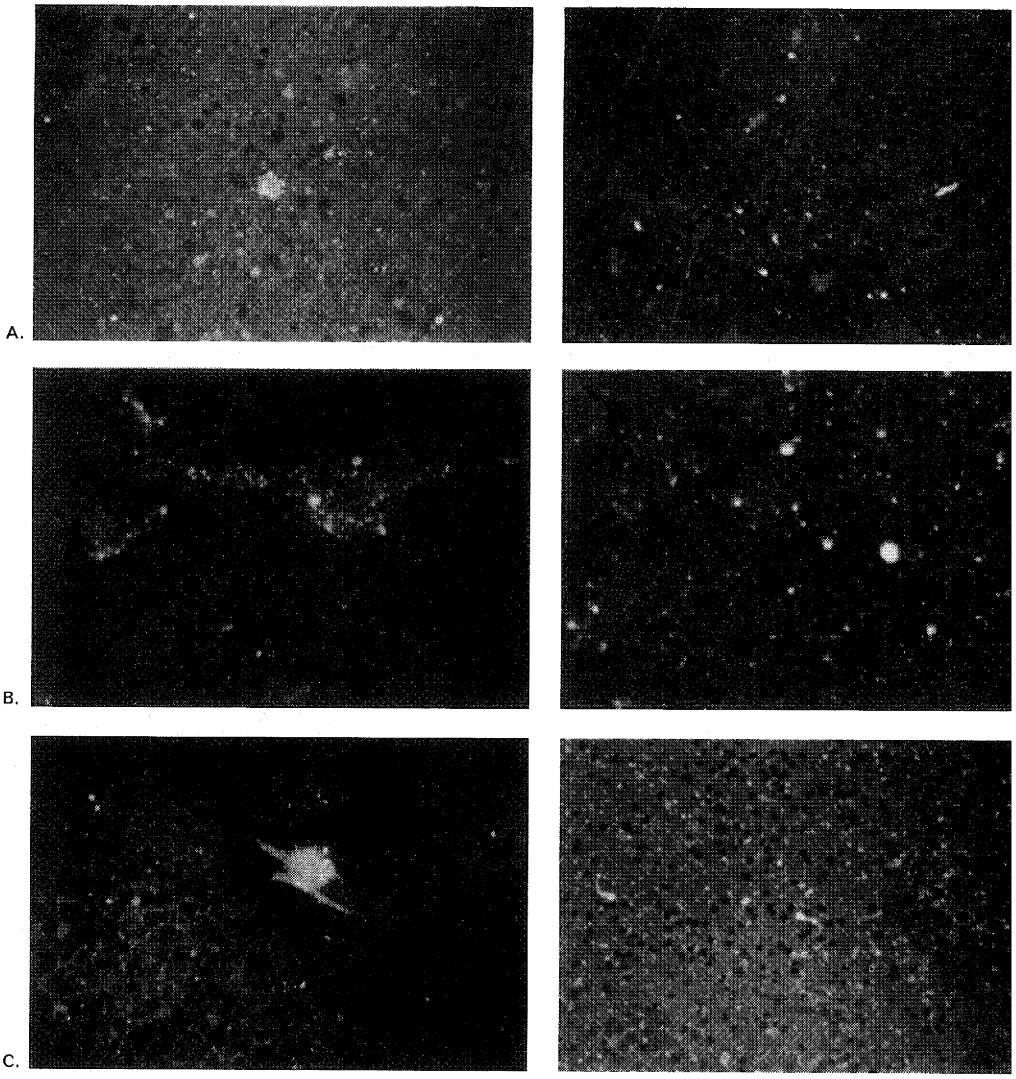
Fig. 7.1 Direct method of detecting rabies antigen



WHO 94920

Schematic representation of reaction of unlabelled rabies virus (antigen) with fluorescein-tagged antibody to yield fluorescein-labelled antigen–antibody product.

Plate 7.1 Photomicrographs of brain material treated with hamster conjugat diluted 1:30



A. Cerebellum—cow. Note fine particles (dust) as well as antigen in nerve cell.
B. Neuron—dog.
C. Neuron—cow.
D. Hippocampus—fox. Typical variation in size. Suitable as positive control slide.
E. Ammon's horn—cat.
F. Cerebellum—mouse (CVS strain).

film, either by rotating the slides or with an applicator stick or toothpick. A fresh stick should be used for each area stained. The slides are placed on a rack in a pan (a shallow boiling pan with suspended glass rods similar to that used for bacterial staining is suitable). Water is added to the pan, which is then covered and placed in an incubator at 37 °C for 30 minutes. This procedure creates sufficient humidity to prevent drying of the test material.

After incubation the slides are washed by dipping in PBS, pH 7.4, then further rinsed by immersing for two successive 10-minute periods. Coplin jars are convenient for this purpose. The slides are then removed and air-dried in a vertical position. When dry, one drop of 50% buffered glycerol (pH 7.6) is added and coverslips are placed over the areas to be examined. When properly stained, the positive control film and test films containing rabies antigen will contain brilliantly fluorescing apple-green or greenish-yellow structures, varying in size from tiny bodies, which are commonly called sand or dust and are barely visible, to those comparable in size to Negri bodies (Plate 7.1). Fluorescing antigen of diagnostic significance may be seen in nerve fibres and cells from both the central nervous system and the salivary glands. Control slides should *always* be examined before and after scanning the test films to ensure that the equipment is operating satisfactorily and that films are properly stained. When large numbers of slides are examined consecutively, control slides should also be interspersed with the test slides, e.g. every tenth slide.

No fluorescence should be seen on films or smears prepared from conjugate diluted with uninfected mouse-brain suspension as described above; this control procedure is therefore important in determining the specificity of the fluorescence and minimizing the number of false positives.

A number of techniques are available for the *intra vitam* diagnosis of rabies in humans and animals. Viral antigen may be detected by the FA test in corneal impressions, skin biopsies or saliva samples from patients with rabies. In addition, the test may be used to detect rabies virus-neutralizing antibodies in serum samples from patients with rabies. However, the technique is of limited sensitivity for *intra vitam* diagnosis since a negative result does not rule out the possibility of infection. Accordingly, samples diagnosed as negative should be inoculated into suckling mice (see Chapter 6).

Discussion

The FA test for the detection of rabies antigen has been perfected to such an extent that it is one of the quickest and most reliable methods available, both for diagnostic and research purposes. Errors can usually be traced to faulty equipment, unsatisfactory conjugate, lack of control slides, and lack of experience in reading slides.

The FA test may occasionally yield positive results when the mouse inoculation test is negative, since the former detects inactivated as well as live antigen. In this respect, fluorescence tests are more sensitive than inoculation tests in mice. All test slides submitted to 50 state health department laboratories in the USA in 1968 were diagnosed correctly. Some laboratories have reported, on occasion, mouse-positive, fluorescent-negative specimens, but such reports have declined as staff have gained experience in performing the FA test.

From 1966 to 1970, nearly 15 000 specimens were examined in the Laboratories for Veterinary Science of the New York State Department of Health, Albany, NY, USA. Of these, 802 of a total of 804 were found positive by the FA test. In only 2 cases was the mouse test positive and the FA test negative. Insufficient sampling was responsible for these results. Since samples found positive in the FA test are not routinely injected into mice, data are not available on the reverse situation. It is perhaps significant that the New York and Californian state laboratories, which reported almost perfect correlation, employed a conjugate of hamster origin that could be diluted as much as 1:50 prior to use, thus minimizing or eliminating the problem of nonspecific fluorescence. It is advisable to test in mice all fluorescent-negative specimens involving human exposure in order to monitor the accuracy of the FA test. It should be kept in mind that suckling mice (less than 3 days old) are more susceptible to rabies than weanling or adult mice and therefore should be used where rapid results are important. Since antigen can be detected by the FA test as early as 3 days after inoculation, one mouse per day can be killed beginning on day 3.

Errors may also result from the use of unsatisfactory or inadequately adjusted equipment, nonspecific fluorescence, gradual and unrecognized loss of light from a fluorescent lamp, failure to use the adequate controls, and colour blindness on the part of the operator. In addition, care should be taken to ensure that cross-contamination of slides does not occur.

Adequate sampling is an important factor. Since rabies virus is usually transmitted from the site of inoculation to the central nervous system via the peripheral nerves, we routinely examine brain stem material either separately or as part of the composite sample. On repeated occasions, the brain stem has been positive when films prepared from the hippocampus, cerebellum or cerebral cortex were negative. Brain-stem sampling is particularly advantageous when animals are killed during the early stages of the disease.

With satisfactory conjugate, counterstains such as rhodamine-conjugated albumin are rarely needed. When rhodamine is used in dilutions of less than 1:80, loss of specific fluorescence occurs in direct proportion to the concentration of rhodamine used. Dilutions of 1:80 or greater are sufficient to stain background material a light brick red, leaving the greenish-yellow rabies antigen in sharp contrast. There is no apparent correlation between the condition of the specimen examined and the degree of nonspecific fluorescence. Good to excellent fluorescence with little or no nonspecific fluorescence has been observed in badly decomposed specimens and, occasionally, is encountered in fresh specimens. With experience, however, it is possible to differentiate between specific and nonspecific fluorescence without great difficulty. The importance of using inhibited conjugate (diluted as described earlier) cannot be overemphasized since this procedure will eliminate nonspecific fluorescence.

Examination of the salivary glands should be made routinely in the case of bats involved in human exposure and may yield valuable information with other species as well. Salivary glands are more difficult to sample adequately and to process than brain material. Since grinding with a pestle and mortar is not usually satisfactory owing to the character of the tissue involved, films are customarily prepared from the salivary-gland tissue by the impression technique (see page 57) and stained in the same way as brain material used for the direct microscopic test.

The fluorescence seen in salivary-gland preparations is more diffuse than that seen in brain tissue. Nonspecific fluorescence, varying from blue to red, is often seen and appears to be especially characteristic of glandular material. With adequate sampling and routine use of control slides, examination of the salivary glands by the FA test can provide accurate information for the diagnosis of rabies in bats.

CHAPTER 8

Virus isolation in neuroblastoma cell culture

W. A. Webster[1] *& G. A. Casey*[2]

Techniques for the isolation of field strains of rabies virus in cell culture are now well developed and are widely used for the diagnosis of rabies. In many laboratories, the rabies tissue-culture infection test (RTCIT) has replaced the mouse inoculation test (MIT). The RTCIT is relatively easy to perform, is much less expensive than the MIT and, most important, can substantially reduce the time required for obtaining results.

The early use of cell cultures for the isolation and growth of various rabies virus strains was reviewed by Crick & King (*1*) in 1988. The first attempts at isolating field virus used baby hamster kidney (BHK-21) cells. However, the results were not encouraging, since the sensitivity of this cell type was often less than that of the MIT. Numerous other cell types, including chick embryo-related (CER), McKoy, bovine, bat, skunk, dog and racoon cells have been used for the isolation of rabies field virus (*2–4*). While BHK-21 cells are still used in certain serological tests and in studies on fixed rabies virus strains, murine neuroblastoma cells are now generally accepted as being superior to all others tested for the isolation of field virus strains (*4, 5*).

Before any new technique can be accepted as a recognized diagnostic test, its sensitivity must be rigorously compared with that of established tests. Numerous comparisons have now been made between the RTCIT and both the fluorescent antibody (FA) test and the MIT (*3, 5–9*). Results of these studies indicate that the RTCIT is at least as sensitive as the MIT in demonstrating the presence of rabies field virus in animal and human tissues. In studies with inocula containing very small amounts of field virus, the MIT in 21-day-old mice demonstrated virus in only 50%, whereas the RTCIT detected virus in 99% of the same inocula (*9*).

The RTCIT procedure outlined below has been developed to enable a small number of staff to examine a relatively large number of specimens (*9*). Between June 1986 when the test was formally adopted by the rabies unit of the Animal Diseases Research Institute and December 1990, 52 600 specimens suspected of being infected with rabies were examined by a staff of five. A total of 27 500 specimens diagnosed as negative by the FA test were tested by the RTCIT and 34 of these were found to be positive. Under these conditions, the test must be designed in such a way that it is easily performed, reliable and inexpensive. The RTCIT is as quick to perform as the MIT and the costs associated with the latter are up to 5 times higher. Most importantly, results are obtained within 4 days with the RTCIT, as compared with 30 days in the MIT (see also Chapter 6).

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Rabies tissue-culture infection test (RTCIT)

Tissue preparation

Brain tissue is mashed on a paper towel using a wooden tongue-depressor and approximately 0.5 g is added to 5 ml of phosphate-buffered saline (PBS) containing antibiotic (see Annex 1) to make a 10% (w/v) suspension. This suspension is shaken or vortex-mixed vigorously and allowed to settle for at least 1 hour at 4 °C. The upper clear layer is then withdrawn and diluted 10-fold with Eagle's minimum essential medium supplemented with 10% fetal calf serum (EMEM-10) (see Annex 1) to make a 1% (w/v) suspension.

Cell suspension

Murine neuroblastoma (NA-C1300) cell cultures are grown at 35–36 °C in 25-cm^2 plastic culture flasks containing 5 ml of EMEM-10. A flask with a nearly confluent monolayer will provide sufficient cells to inoculate one 96-well microtitration plate. Monolayers are detached by trypsinization and resuspended in EMEM-10 to give a concentration of approximately 5×10^5 cells/ml. Immediately before use, diethylaminoethyl-dextran (DEAE-dextran) is added to give a final concentration of 25 μg/ml.

Test procedure

1. For each specimen, add 0.1 ml of the cell suspension to each of 4 wells of a 96-well microtitration plate. A total of 24 specimens can be tested on one plate.
2. Add 0.2 ml of the 1% brain-tissue suspension (in EMEM-10) to each of the 4 wells containing cells.
3. Mix the wells on a mixer.
4. Incubate at 35–36 °C in a humid chamber with 5% carbon dioxide (CO_2) for 4 days. The plates should be checked daily for cell growth. If contaminated wells are noted, that specimen should be re-mixed in saline containing twice the original amount of antibiotic and re-inoculated into new cell cultures.
5. Prepare a positive field virus (3 wells) and negative (2 wells) control plate. This should be done on a separate microtitration plate and after all the test plates have been prepared in order to eliminate any possibility of contaminating a negative specimen with rabies-positive material.
6. After incubation, remove the media from the wells and wash once with 0.01 mol/l PBS (Annex 1).
7. Fix the cells by adding cold 70–80% acetone to each well and incubate at room temperature for 30 minutes.
8. Shake out the acetone and air-dry the plates.
9. Add 1 drop of fluorescein–antibody conjugate to each well and incubate at 37 °C for 30 minutes.
10. Shake out the conjugate and wash once with 0.01 mol/l PBS.
11. Counterstain with Evans Blue (1:40 000) for 15–30 seconds.
12. Rinse once with 0.01 mol/l PBS.
13. Examine the plates under an ultraviolet microscope using a 10× objective.

Factors affecting sensitivity

Various factors such as specimen contamination, preservation and storage may affect the sensitivity of the RTCIT. These are discussed briefly below.

Cell culture

Various strains of field rabies virus (as identified with monoclonal antibodies) may differ in biological properties such as their isolation and growth characteristics in cell cultures. While murine neuroblastoma cells have been shown to be more sensitive than BHK-21 cells to infection by most field viruses, some virus strains grow equally well in both cell types (*10*). Moreover, not all neuroblastoma cell lines are equally sensitive (*11*). Differences have also been noted between cultures of the same cell line obtained from different sources (W.A. Webster, unpublished observation). In the latter study, two cultures of murine neuroblastoma cells (NA-C1300) were obtained, each from a different laboratory, and inoculated with 10% brain suspensions of the Canadian Arctic field strain (*12*). Results indicated average infection rates of 65% and 10% with the same inocula in the two cultures. Before setting up a diagnostic procedure, extensive testing must therefore include the comparison of cell cultures from various sources.

Tissue preparation

The 10% brain-tissue suspensions can be clarified either by allowing the larger debris to settle or by centrifugation. Depending upon various factors, centrifugation of the suspension may reduce the amount of virus demonstrable by cell culture (*5*). Allowing the tissue to settle in PBS containing antibiotic for at least 1 hour has several advantages. It reduces the costs of the test, since a centrifuge is not required. In addition, the lack of nutrients and the presence of antibiotics in the saline can eliminate a certain amount of bacterial contamination in the tissue, which is often received in various stages of decomposition. However, the use of saline–antibiotic diluent is not recommended for long-term storage of tissue suspensions. Cell-culture medium containing 10% fetal calf serum (EMEM-10) is fairly effective for preservation of rabies virus (including fixed strains), although some field viruses lose infectivity even at −70 °C.

The use of a further 10-fold dilution in EMEM-10 of the 10% brain suspension as inoculum removes most toxic substances associated with brain tissue. Cell growth is improved and the resulting infection rates are at least as high as those of cultures inoculated directly with the 10% brain suspension (*9*).

While brain is the preferred tissue for testing, other tissues or fluids may be used when there is damage to the cranium or for *intra vitam* diagnosis. Salivary-gland tissue or saliva is often used and virus can be easily isolated in cell culture if serial dilutions in EMEM-10 are used as the inoculum. The use of a 1% tissue suspension is recommended, since inocula containing large amounts of salivary-gland tissue often produce severe cytolysis in cell culture. Rabies virus may also be isolated from spinal-cord tissue, which may be positive when brain tissue is negative, as well as from fluids (saline) used to wash the cornea, although trials with the latter have been limited (*4*).

Specimen contamination

Bacterial and fungal contaminants can have a deleterious effect on cell cultures. The preparation of tissue suspensions in cold PBS containing antibiotic reduces

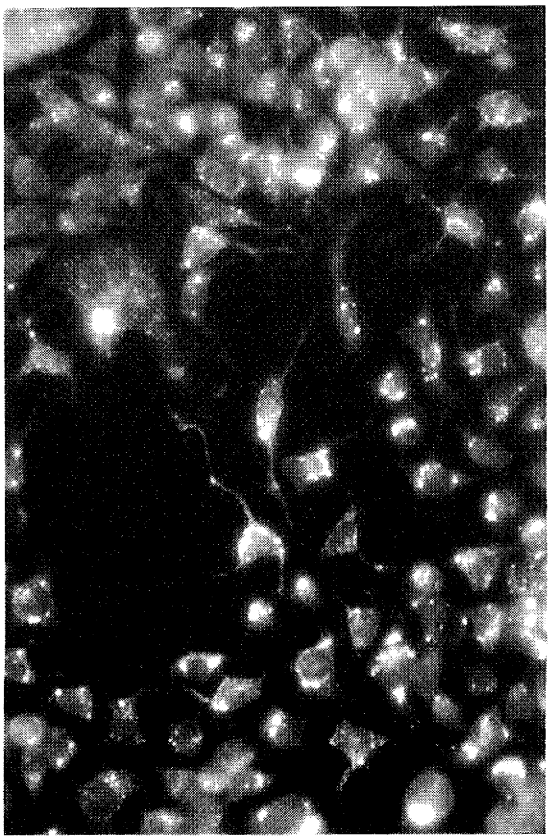
the effects of bacterial contaminants (*9*). Of some 3700 specimens tested during a period when ambient temperatures ranged between −10 °C and +30 °C, only 4 of these were unfit for a diagnosis to be reached. In severely contaminated cases, extra antibiotic may be added. A fungicide may also be added to control fungi, although growth of these organisms does not always result in cell destruction.

Filtration of tissue suspensions to remove contaminants reduces the amount of demonstrable virus; in some cases, the number of positive cells may be reduced by 50–100% (W.A. Webster & G.A. Casey, unpublished observation).

Test plates

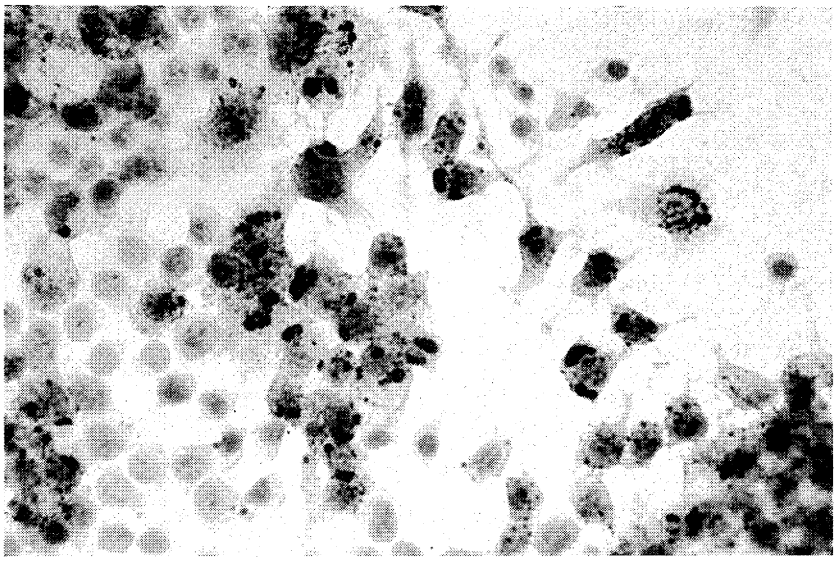
The RTCIT can be performed on 96-well microtitration plates (*5*, *9*), 60-well or 96-well human leukocyte antigen (HLA) plastic plates (*3*), coverslips (*8*), or on multi-chambered glass slides (*6*, *7*). The use of 96-well microtitration plates is preferred to other types for a variety of reasons. Although the volume (13 μl) of suspension used in each well of an HLA plate is sufficient for suspensions containing large amounts of virus, the results obtained with suspensions containing very small

Fig. 8.1 Rabies-infected neuroblastoma cells stained by immunofluorescence



× 100

Fig. 8.2 Rabies-infected neuroblastoma cells stained by the avidin–biotin technique



amounts of virus (as would be expected in these test suspensions) are inconsistent. Coverslip cultures grown in flasks pose special problems with handling because of their fragility. The use of multi-chambered glass slides can increase the cost of the test by a factor of 5–10.

Fixation and staining

Fixation of cell monolayers can be accomplished using either acetone, various alcohols or formalin. Both 70–80% acetone and an absolute methanol:formalin (1:1) mixture have been used on plastic microtitration plates and produce excellent results (*5*). Formaldehyde (10% solution) may also be used, but tends to diminish fluorescence when the virus is stained with immunofluorescent stains.

The usual method of examining cell cultures for the presence of rabies virus is by immunofluorescence staining. Counterstaining with Evans Blue increases the contrast by decreasing background and nonspecific fluorescence. Care must be taken, however, not to overstain with Evans Blue since it can also reduce specific fluorescence. The use of a 100 × magnification (10 × objective) gives excellent results (Fig. 8.1) and allows a much more rapid examination than at higher magnifications. Staining cells by immunoenzymatic (*13, 14*), peroxidase–antiperoxidase (*15*) and avidin–biotin (Fig. 8.2) methods also gives satisfactory results (A. Bourgon, unpublished results), but is more time-consuming and more technically complicated (see Annex 2). Infected monolayers on glass slides can be stained with histological stains (see Chapter 4), such as van Giesen's stain for Negri bodies; however, this method is not recommended, since only the larger inclusion bodies are seen and many moderate to light infections would be misdiagnosed.

Incubation period

It is now generally accepted that the test culture should be incubated for 4 days (*5*, *9*). Shorter periods have been suggested (*3*, *6–8*). However, when only small amounts of virus are present, a longer incubation period is required. Most specimens which are negative by the FA test and subsequently positive by the RTCIT infect only small numbers (1–5%) of cells in culture (*9*). Examination of these cultures on successive days post-infection indicated that a positive diagnosis would have been made in only half of the specimens examined at 2 days, whereas all cultures were rabies-positive at 4 days post-infection (*9*).

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Annex 1

Media

Eagle's minimum essential medium (EMEM), as modified by MacPherson & Stoker, 1962

Component	*mg/l*
Sodium chloride (NaCl)	6800.0
Glucose	1000.0
Potassium chloride (KCl)	400.0
Calcium chloride ($CaCl_2$)	200.0
Sodium phosphate, monobasic, monohydrate ($Na_2H_2PO_4 \cdot H_2O$)	140.0
Magnesium sulfate ($MgSO_4$)	97.7
Phenol red	17.0
Sodium succinate, hexahydrate	100.0
Succinic acid	75.0
L-Arginine hydrochloride	126.4
L-Disodium cystine	28.4
L-Histidine hydrochloride, monohydrate	41.9
L-Isoleucine	52.5
L-Leucine	52.5
L-Lysine hydrochloride	73.1
L-Methionine	14.9
L-Phenylalanine	33.0
L-Threonine	47.6
L-Tryptophan	10.2
L-Tyrosine	36.2
L-Valine	46.9
Calcium D-pantothenate	1.0
Choline bitartrate	1.8
Choline chloride	1.0
Folic acid	1.0
i-Inositol	2.0

Nicotinamide	1.0
Pyridoxal hydrochloride	1.0
Riboflavin	0.1
Thiamin hydrochloride	1.0

EMEM-10

EMEM-10 is prepared from EMEM, as above, supplemented with:

Fetal calf serum	10%
Tryptose phosphate broth	10%
1-Glutamine	2 nmol/l
Neomycin sulfate	50 μg/ml

Phosphate-buffered saline (PBS), 0.01 mol/l, pH 7.4

Sodium chloride (NaCl)	150 g
Sodium phosphate, dibasic (Na_2HPO_4)	20.5 g
Potassium phosphate, monobasic (KH_2PO_4)	4.9 g
Distilled water to make	18 litres

The pH should be adjusted to 7.4 by adding either Na_2HPO_4 or KH_2PO_4 as required.

PBS with antibiotic

PBS with antibiotic is prepared from PBS, as above, supplemented with:

Streptomycin sulfate	2 mg/l
Benzylpenicillin	1000 IU/ml

Annex 2
Avidin–biotin staining method[1]

1. Fix cell cultures in cold 70–80% acetone and incubate at room temperature for 30 minutes. Shake out acetone and air-dry.
2. Rinse each well (3 times) with tromethamine[2] buffer (0.01 mol/l, pH 7.5).
3. Cover cells with normal goat serum diluted 1:20 with tromethamine buffer and incubate at 45 °C in a humid chamber for 20 minutes.
4. Repeat step 2.
5. Cover cells with primary antisera (rabbit anti-N protein serum diluted in tromethamine buffer, preferably at a high dilution if titre permits) and incubate at 45 °C for 20 minutes. Rinse with tromethamine buffer and repeat incubation.
6. Repeat step 2.
7. Cover cells with bridge or link antisera (biotinylated goat antirabbit serum diluted 1:20 in tromethamine buffer) and incubate at 45 °C for 20 minutes.

[1] W.A. Webster, unpublished.

[2] Also known as tris(hydroxymethyl)aminomethane.

8. Repeat step 2.
9. Immerse cells in 30% hydrogen peroxide (H_2O_2) diluted 1:25 with absolute methanol and leave at room temperature for 10 minutes. Repeat. (No rinse between.)
10. Repeat step 2.
11. Cover cells with ABC complex (streptavidin-biotinylated horseradish peroxidase) and incubate at 45 °C for 20 minutes. This should be prepared 30 minutes in advance (mix 0.1 ml of streptavidin with 10 ml of tromethamine buffer and then add 0.1 ml of biotinylated peroxidase).
12. Repeat step 2.
13. Cover cells with fresh diaminobenzadine tetrahydrochloride (DAB) solution (0.05% DAB in tromethamine buffer (10 ml) with 3 μl of H_2O_2) and leave at room temperature for 2 minutes. Mix immediately before use (at late stage of ABC complex incubation). Repeat.
14. Rinse with tromethamine buffer.
15. Rinse with distilled water.
16. Counterstain with haematoxylin for 1 minute and change to fresh haematoxylin for 5 minutes. Wash twice in tap water. Blue in tromethamine buffer.
17. Dehydrate rapidly (to absolute alcohol), treat with xylene, and mount.

CHAPTER 9

Rapid rabies enzyme immunodiagnosis (RREID) for rabies antigen detection

H. Bourhy[1] *& P. Perrin*[2]

Introduction

Enzyme immunoassays have recently replaced many of the traditional techniques for diagnosing viral infections in animals and humans. These new procedures do not require expensive equipment and are quick and easy to perform. Moreover, they are considerably safer than many of the traditional techniques and, because of their high sensitivity and specificity, can also be applied to large numbers of specimens under field conditions. In 1986 a solid-phase enzyme-linked immunosorbent assay (ELISA) called rapid rabies enzyme immunodiagnosis (RREID) was developed for the diagnosis of rabies, based upon the detection of rabies virus nucleocapsid antigen in brain tissue (*1*). During extensive trials, it was found easy to perform and showed high precision and reproducibility (*2–6*). Subsequently, an RREID test using enzyme-labelled avidin–biotin (RREID-biot) was developed. It appeared to increase slightly the sensitivity of the RREID without affecting its specificity. However, the most recently developed enzyme immunoassay (RREID-lyssa), also based on an avidin–biotin amplification system, further increases the detectability of rabies-related strains (lyssavirus serotype 2, 3 and 4 and European bat lyssavirus) in infected specimens (*7*).

Method

Principle of the RREID

Rabies nucleocapsid antigen is extracted from infected cells and purified on a caesium chloride (CsCl) gradient (*8, 9*). The viral antigen is emulsified in Freund's adjuvant and administered intramuscularly to outbred rabbits. After several injections, blood samples are taken from the rabbits. Rabies antinucleocapsid IgG is then purified from the serum using chromatography (*10*) (see also Appendix 2).

The immunoassays described here employ rabies antinucleocapsid rabbit IgG as solid phase (*1*). However, the RREID-lyssa uses a mixture of rabbit IgG directed against lyssavirus serotype 1 (PV strain), serotype 3 (Mokola virus) and European bat lyssavirus (EBL1), while the RREID and RREID-biot are performed with IgG directed against only the PV strain. The incubation of a positive specimen results in the binding of rabies nucleocapsid antigen to the IgG. In the RREID, the bound viral antigen is revealed with a peroxidase conjugate (rabies antinucleocapsid rabbit IgG conjugated with horseradish peroxidase), while the RREID-biot and RREID-

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lyssa employ an IgG–biotin conjugate (biotinylated rabies antinucleocapsid rabbit IgG conjugated with a streptavidin–peroxidase complex (*7*)) (Fig. 9.1). A yellow-orange colour appears after the addition of the substrate and *o*-phenylenediamine (chromogen); negative specimens are colourless. In both tests, the coloration can be evaluated qualitatively, with the naked eye (Fig. 9.2), or quantitatively, using a spectrophotometer (Fig. 9.3).

Safety precautions

The isolation of rabies nucleocapsid antigen should preferably be performed according to Biosafety Level 3, particularly when rabies-related strains are used (*11*). All specimens and the positive antigen control should be regarded as potentially virulent, even after they have been inactivated by β-propiolactone or by heating. They should therefore be carefully handled with gloves and fluids should be collected in a recipient containing 5% sodium hypochlorite (bleach) solution. All chemicals should be used according to good laboratory practice and any contact with skin or mucosae should be avoided.

Preparation of reagents

Rabies antinucleocapsid rabbit IgG

The techniques for producing and purifying rabies antinucleocapsid rabbit IgG directed against rabies serotype 1 (PV strain), serotype 3 (Mokola virus) and

Fig. 9.1 Principle of the RREID, RREID-biot and RREID-lyssa

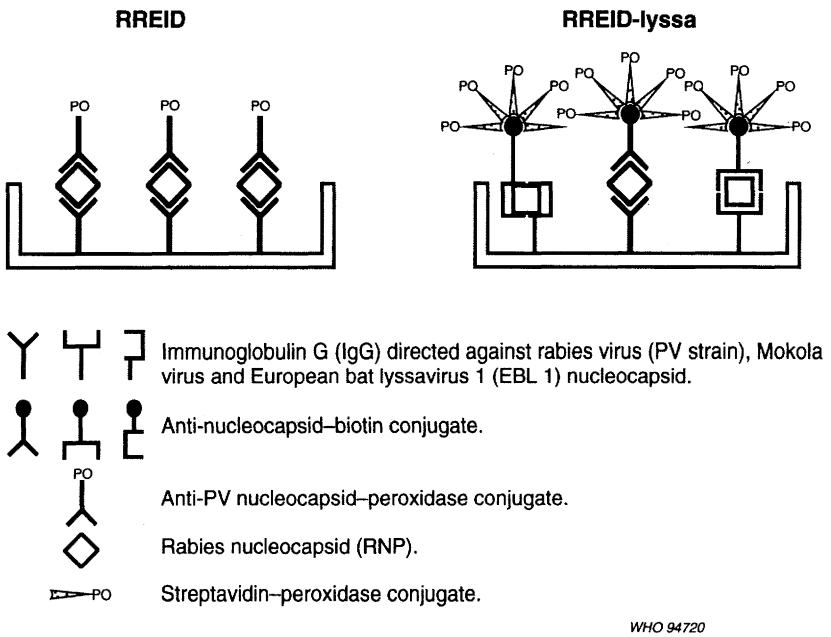
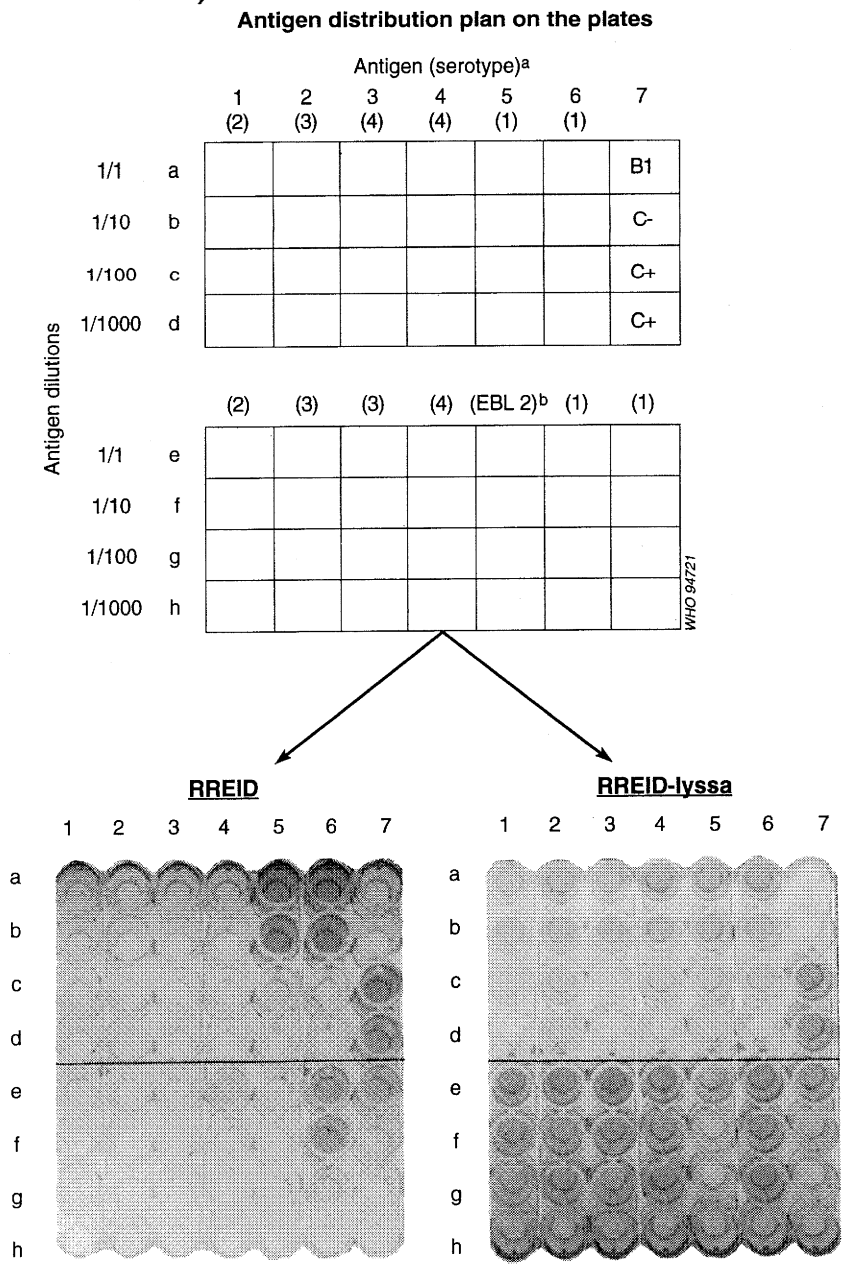


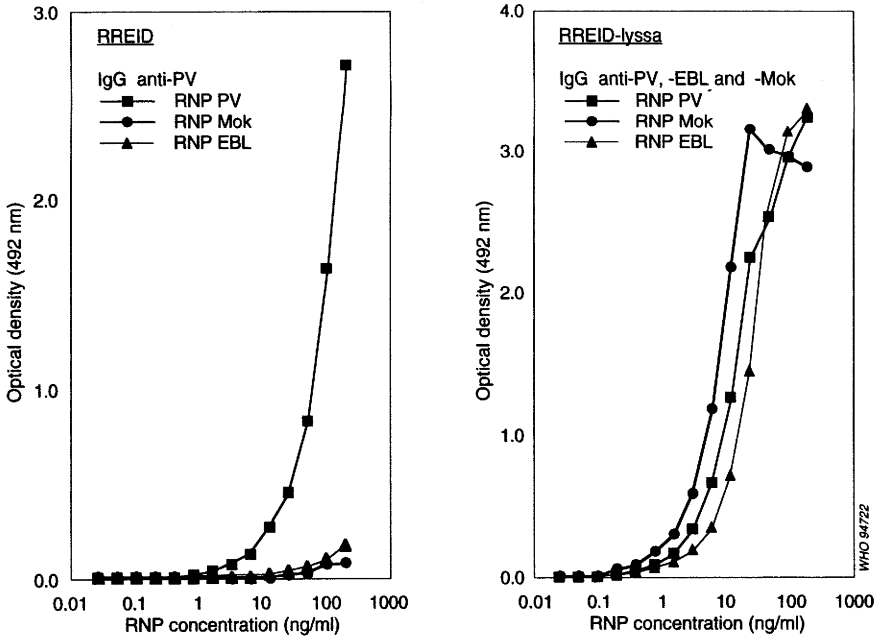
Fig. 9.2 Diagnosing rabies by the RREID and RREID-lyssa (qualitative method)



a Serotype 1 refers to rabies virus (PV strain); serotypes 2, 3 and 4 refer to Lagos bat, Mokola and Duvenhage viruses respectively.

b Refers to European bat lyssavirus 2.

Fig. 9.3 Diagnosing rabies by the RREID and RREID-lyssa (quantitative method)



PV = rabies virus (PV strain).
Mok = Mokola virus.
EBL = European bat lyssavirus.

European bat lyssavirus (EBL1) and conjugating the rabbit IgG to peroxidase or biotin are described in Appendix 2.

Sensitization of microtitration plates

Microtitration plates with 96 wells divided into 6 strips of 16 wells each should be used. They should be sensitized as follows:

1. Add 1.0 µg (200 µl) of purified rabies antinucleocapsid IgG in carbonate buffer, pH 9.6, to each well. Incubate for 3 hours at 37 °C.
2. Add 300 µl of 0.3% bovine serum albumin and 5% sucrose in carbonate buffer, pH 9.6, to each well and incubate for a further 30 minutes.
3. Vacuum-dry the microtitration plates and store at − 20 °C. Alternatively, plates that are sealed under vacuum may be stored at 4 °C.

Preparation of solutions

The preparation of a stock solution of PBS–polysorbate, pH 7.4 for the washings is described in the Annex. The buffer for peroxidase substrate and the stopping solution (4 mol/l sulfuric acid) for the enzymatic reaction are made ready for use.

Preparation of control antigens

1. Remove the brains of uninfected (negative control) and rabies-infected (positive control) mice under aseptic conditions and homogenize in PBS, pH 7.0, 10-fold concentrate (PBS 10X).
2. Centrifuge the brain suspensions for 30 minutes at 2000 *g* and inactivate the supernatants by adding β-propiolactone to a final concentration of 1 : 4000 and incubate for 24 hours at 4 °C. The control suspensions may be stored at − 20 °C or (preferably) lyophilized in 1-ml aliquots. The rabies strain used for the positive control should belong to the same serotype as that against which the conjugate is directed.

Preparation of samples

1. Homogenize a sample of the brain stem, cortex and Ammon's horn from each brain submitted for diagnosis at 30% (w/v) either in PBS 10X, pH 7.4, or in cell-culture medium at 4 °C (if virus isolation in cell culture is also performed on the same specimen; see Chapter 8). The test suspension may also be prepared from brain specimens collected by the retro-orbital or occipital foramen routes (*12*) (see also Chapter 4 and Appendix 2).
2. Centrifuge the suspension for 30 minutes at 2000 *g*.
3. Inactivate the supernatants by heating in a water bath for 2 hours at 56 °C.

Performing the test

Reconstitution of reagents

1. Open the vacuum pack containing the sensitized microtitration plates and take out the required number of plates. Reseal the pack and keep it at 4 °C.
2. Wash the strips 5 times in PBS–polysorbate, pH 7.4 and dry on absorbent paper at room temperature.
3. Reconstitute the negative and positive control antigens, if they are lyophilized, using 1 ml of distilled water.
4. Dilute the required quantity of conjugate 1:10 in PBS 10X, pH 7.4.
5. Prepare the substrate—*o*-phenylenediamine (chromogen) solution—just before use and keep it in the dark.
6. Place all the reagents at room temperature approximately 10 minutes before starting the test.

Immunocapture

1. Prepare a blank control for the photometric readings by adding 200 µl of PBS–polysorbate–bovine serum albumin (BSA) to the first well (1A) of the microtitration plate (in some automatic plate readers, a blank control should be made in all the wells of the first line, 1A–1H).
2. Add 200 µl of the positive and negative control antigens to the second and third wells (1B and 1C).
3. Add 200 µl of the supernatant of each sample to the remaining wells.
4. Seal the microtitration plate with adhesive plastic film and incubate for 1 hour at 37 °C.
5. Remove the film and aspirate the contents of each well into a recipient containing 5% sodium hypochlorite (bleach) solution.

6. Fill the wells with washing solution (PBS–polysorbate). Invert the microtitration plate and blot onto absorbent paper. Repeat the procedure 5 times.

RREID

1. Add 200 µl of peroxidase conjugate to each well.
2. Seal the microtitration plate with adhesive plastic film and incubate for 1 hour at 37 °C.
3. Remove the film and aspirate the contents of each well.
4. Fill the wells with washing solution. Invert the plate and blot onto absorbent paper. Repeat the procedure 5 times.
5. Add 200 µl of the substrate–chromogen solution to each well. Leave the microtitration plate in the dark for about 20 minutes at room temperature.
6. Add 50 µl of stopping solution to each well. Do not expose the microtitration plate to direct light before reading.

RREID-biot and RREID-lyssa

1. Add 200 µl of IgG–biotin conjugate to each well.
2. Seal the microtitration plate with adhesive plastic film and incubate for 1 hour at 37 °C.
3. Remove the film and aspirate the contents of each well.
4. Fill the wells with washing solution. Invert the plate and blot onto absorbent paper. Repeat the procedure 5 times.
5. Add 200 µl of IgG–biotin conjugate to each well and incubate for 30 minutes at 37 °C.
6. Aspirate the contents and repeat step 4.
7. Add 200 µl of substrate–chromogen solution to each well and leave the microtitration plate in the dark for 10 minutes at room temperature.
8. Add 50 µl of stopping solution to each well. Do not expose the microtitration plate to direct light before reading.

Interpretation of results

Reading with the naked eye

The colour can be easily evaluated with the naked eye. The blank and the negative antigen control should appear colourless, while the positive antigen control should be yellow-orange. Specimens that show an orange coloration are considered positive, while those that are colourless are considered negative. This reading is often sufficient for diagnostic purposes.

Reading with a spectrophotometer

Absorbency measurements should be taken within 30 minutes of stopping the reaction (see above, step 8).

1. Carefully wipe the bottom of the microtitration plate and place it in the spectrophotometer.
2. Determine the optical density (OD) at 492 nm of the blank(s), controls and samples.
3. Calculate the absorbency by subtracting the OD of the blank from that of the controls and samples. The absorbency of the negative and positive antigen controls should be below 0.1 and above 1.5 units respectively, otherwise the test

is not valid. Samples that have an absorbency of more than 0.08 units (in the case of the RREID) or 0.1 units (RREID-biot and RREID-lyssa) above that of the negative control are considered positive.

Evaluation of the technique

The RREID has been evaluated in various epidemiological situations. When used for the routine diagnosis of rabies on some 10 000 specimens collected in France, the sensitivity and specificity of the technique were 98.5% and 99.9%, respectively (*3*). The results obtained under field conditions by 17 laboratories in Africa, America, Asia and Europe also showed a good correlation, especially considering that the technique was being performed for the first time (*2*, *3*). One major characteristic of the RREID is that its reliability is not affected by decomposition or heat inactivation of the specimen. Furthermore, testing by RREID of brain specimens collected through the occipital or retro-orbital foramen routes provides a very useful tool for epidemiological surveys of rabies (*12*). The detection limit of nucleocapsid antigen of lyssavirus serotype 1 is in the range of 0.8–1.0 ng/ml for RREID. Nevertheless, the sensitivity is lower when specimens infected with rabies-related strains are tested.

To further increase the sensitivity of the immunocapture of rabies antigen, an enzyme-labelled avidin–biotin method was developed. This amplified technique, called the RREID-biot, increases the detection limit of rabies nucleocapsid antigen (serotype 1) to 0.2 ng/ml without affecting the specificity. Similarly, a mixture of rabbit IgG directed against serotype 1 (PV strain), serotype 3 (Mokola virus) and European bat lyssavirus (EBL1) can be used for the immunocapture and for the conjugate to increase the detectability of rabies-related strains. This technique, called the RREID-lyssa, increases the detection limit of nucleocapsid antigen to 0.2 ng/ml, whatever the serotype of the strain considered (*7*).

In conclusion, the RREID and especially the RREID-biot and RREID-lyssa tests are excellent tools for the detection of all the currently known lyssaviruses relevant to public health. They are simple, rapid, sensitive, specific and economical. Use of these tests can be recommended either for confirming the results of the fluorescent antibody test or for epidemiological surveys in association with simple methods of brain collection such as the retro-orbital or occipital foramen procedures (*12*). They can also be easily applied to the quantification of rabies antigen.

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Annex

Preparation of reagents

Carbonate buffer, pH 9.6

Sodium bicarbonate ($NaHCO_3$)	4.20 g
Sodium carbonate (Na_2CO_3)	5.30 g
Distilled water to make	1000.00 ml

Phosphate-buffered saline (PBS), pH 7.4, 10-fold concentrate (PBS 10X)

Sodium chloride (NaCl)	80.00 g
Potassium chloride (KCl)	2.00 g
Sodium phosphate, dibasic, dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)	11.33 g
Potassium phosphate, monobasic (KH_2PO_4)	2.00 g
Distilled water to make	1000.00 ml

PBS–polysorbate, pH 7.4

PBS 10X	100.0 ml
Polysorbate 20	200.5 ml
Distilled water to make	1000.0 ml

PBS–polysorbate–BSA, pH 7.4

PBS 10X	10.00 ml
Polysorbate 20	200.05 ml
Bovine serum albumin (fraction V)	0.50 g
Distilled water to make	1000.00 ml
(pH adjusted to 7.4 using 4 mol/l sodium hydroxide)	

Buffer for the peroxidase substrate, pH 5.6 (citrate buffer)

Sodium citrate, dihydrate	11.67 g
Citric acid, monohydrate	2.17 g
Hydrogen peroxide, 30% solution (H_2O_2)	1.00 ml
Distilled water to make	1000.00 ml

Substrate–chromogen solution

o-Phenylenediamine	50 mg
Citrate buffer (prepared as above)	25 ml

CHAPTER 10

Cell culture of rabies virus

A. A. King[1]

The rabies virus is highly neurotropic, but in the infected animal after replication in the central nervous system (CNS) it may spread centrifugally to most organs of the body, in which it is often able to replicate efficiently. It is not surprising, therefore, that the rabies virus can be cultivated in a wide variety of host cells. Such cultivation is extremely important, not only for obtaining knowledge about the virus itself, but also for producing large quantities of virus for the preparation of vaccine.

Susceptible cells, cell lines and strains

Tissue-culture techniques were first applied to the study of rabies virus in 1913 by Noguchi (*1*) and Levaditi (*2*). Levaditi (*2*) also reported the first successful propagation of the virus in spinal ganglia maintained in a medium containing coagulated monkey plasma. In 1930, Stoel (*3*) carried a strain of the virus through five passages in embryonic chicken brains and hearts explanted in rabbit plasma clots without loss of infectivity. The studies with primary cell explants did not, however, provide much information about the kinetics of virus replication.

Atanasiu et al. cultivated street and fixed strains of virus in non-neural cells of a mouse ependymoma tumour cell line and their work led to the first report of intracytoplasmic inclusions resembling Negri bodies in a cell-culture system (*4–6*).

The susceptibility of a variety of primary kidney-cell cultures (of mouse, hamster, pig, dog and monkey origin) and avian embryo fibroblasts to infection with rabies virus demonstrated that these cells could potentially be used for rabies vaccine production. For example, a modification of the first rabies vaccine prepared in primary hamster kidney-cell culture (*7*), incorporating the Beijing virus strain, is used for the preparation of human vaccine in China (*8*) (see also Chapter 30). The Vnukovo-32 strain is used for the production of a similar vaccine in the former USSR (*9*) (see also Chapter 31). In addition, vaccines for human use have been produced from fetal bovine kidney cells (*10*), dog kidney cells (*11*) (see also Chapter 29) and chick embryo fibroblasts infected with Flury LEP C25 rabies virus (*12*) (see also Chapter 27).

Not until the development of several susceptible cell lines and cell strains, however, was a systematic approach to the study of rabies virus and its interaction with host cells made possible. There are now a number of continuous cell lines which are also used for the production of rabies vaccines for animals, such as baby hamster kidney cells, line 21 (BHK-21) (*13*), hamster kidney fibroblasts (Nil-2) (*14*) and chick embryo-related cells (CER) (*15*). Because of the heteroploid

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characteristics and carcinogenic potential of these cell lines, however, only Vero cells have been considered suitable for the production of human vaccines (*16*) (see also Chapter 26).

The production of vaccine in a human diploid cell line (WI-38) was first described in 1964 (*17*). Since then, other human diploid cell strains have been developed, such as human embryonic lung (HEL) (*18*) and human lung (MRC-5) (*19*) (see also Chapter 25), and a rhesus monkey diploid cell line vaccine has also been produced (*20, 21*) (see also Chapter 28).

Rabies virus has been successfully propagated in several cell lines of poikilothermic origin (*22*). It has also been propagated in embryonic chick myotubes, mouse macrophages, rat sensory neurons, rat pituitary tumour cells, black goat and guinea-pig kidney cells, fetal bat cells, human synovial fluid (McCoy) cells, Japanese quail embryo cells and Chinese hamster ovary (CHO) cells (see Table 10.1 for references). In addition, when the relationship between plasma membrane organization and cell susceptibility *in vitro* was investigated in mammalian, avian, fish and arthropod cell lines using CVS virus, specific viral antigen was detected in insect (*Aedes albopictus*) cells, although at a lower level than in the other cells tested (*23*).

Neuroblastoma cells of human or murine origin are now widely used in rabies virus investigations. Mouse neuroblastoma (NA-C1300) cells (hypoxanthine-guanine phosphoribosyl transferase-deficient) are especially useful, since they share a number of characteristics with human neurons, including gross microscopic and fine-structural neuron-like morphology, and the presence of microtubular protein, neurotransmitter synthetic enzymes and electrically excitable cell membranes with acetylcholine receptors (*24, 25*).

Methods of virus propagation

Most conventional methods of virus propagation can be employed for rabies virus propagation in tissue culture. Monolayers in stationary or revolving vessels and in suspension cultures have been used successfully. Some cell types require a specialized medium, but for most purposes the propagation medium is based on Earle's salt solution supplemented with Eagle's amino acids and vitamins or with lactalbumin hydrolysate. Many commercial companies will supply such media either ready to use, or in concentrated or powder form. The addition of serum appears to protect against thermal inactivation of the virus, rather than provide necessary ingredients for virus replication (*26*).

Optimum growth is obtained at a low incubation temperature (32 °C) (*27*) and during virus production the medium should be maintained at a basic pH (7.4–7.6) by the addition of extra sodium bicarbonate (*26*). Open culture systems also require the addition of extra carbon dioxide.

In many cell systems, considerable periods of adaptation and prolonged passaging may be required before substantial yields of virus can be obtained. Diploid cells, such as the normal human diploid fibroblast line WI-38 (*28*) are particularly difficult in this respect, as exemplified by the original adaptation of the Pitman-Moore strain to WI-38 cells (*17, 22, 29, 30*). Prolonged and regular serial passage of infected cells, initially with the addition of fresh uninfected cells, was required before sufficient infectious virus was released into the culture supernatant fluid. This virus was then used to infect naive cells. Even so, the yield of infectious

Table 10.1 Tissue-culture systems for rabies virus

Tissue-culture system	Author(s) and year
Primary cultures	
Mouse kidney cells	Vieuchange et al., 1956 (*87*)
Hamster kidney cells	Kissling, 1958 (*27*)
Pig kidney cells	Abelseth, 1964 (*88*)
Chick embryo fibroblasts	Kondo, 1965 (*89*)
Duck embryo fibroblasts	Kondo, 1965 (*89*)
Dog kidney cells	Hronovsky et al., 1966 (*90*)
Monkey kidney cells	Lang et al., 1969 (*91*)
Fetal calf kidney cells	Atanasiu et al., 1974 (*10*)
Cells of poikilothermic origin	Wiktor & Clark, 1975 (*22*)
Chick embryo myotubules	Lentz et al., 1982 (*75*)
Mouse dorsal root ganglia	Tsiang et al., 1983 (*92*)
Racoon kidney cells	Umoh & Blenden, 1983 (*93*)
Skunk brain cells, skunk kidney cells	Umoh & Blenden, 1983 (*93*)
Japanese quail embryo cells	Seroka et al., 1986 (*94*)
Rat sensory neurons	Lycke & Tsiang, 1987 (*78*)
Rat dorsal root ganglia, rat myotubules	Tsiang et al., 1989 (*95*)
Rat trigeminal ganglion neurinoma	Yusupov et al., 1989 (*96*)
Rat primary cortical neurons	Tsiang et al., 1991a (*97*)
Human dorsal root ganglia	Tsiang et al., 1991b (*98*)
Diploid cell lines	
Human lung WI-38	Wiktor et al., 1964 (*17*)
Human embryonic lung (HEL)	Hronovsky et al., 1973 (*18*)
Rhesus monkey lung IKLL-2	Wallace et al., 1973 (*20*)
Human lung MRC-5	Johnson et al., 1985 (*19*)
Heteroploid cell lines	
Mouse ependymoma cells	Atanasiu & Laurent, 1957 (*4*)
Baby hamster kidney cells, line 21 (BHK-21/S13)	MacPherson & Stoker, 1962 (*13*)
Chick embryo fibroblasts (CEF)	Yoshino et al., 1966 (*32, 33*)
Hamster kidney fibroblasts (Nil-2)	Diamond, 1967 (*14*)
Chick embryo-related (CER) cells	Smith et al., 1977 (*15*)
Green monkey kidney cells, line 4647	Aksenova et al., 1985 (*99*)
Vero monkey kidney cells	Montagnon et al., 1985 (*16*)
Rabbit non-tumour cells	Sureau et al., 1985 (*100*)
Mouse neuroblastoma (NA-1300) cells	Webster, 1987 (*43*)
Mouse neuroblastoma (NA-2) cells	Barrat et al., 1988 (*101*)
Black goat kidney cells	Kim et al., 1988 (*102*)
Guinea-pig kidney cells	Kim et al., 1988 (*102*)
Fetal bat cells	Steece & Calisher, 1989 (*103*)
Rat trigeminal neurinoma (NGUK-1) cells	Yusupov et al., 1989 (*98*)
Human synovial fluid (McCoy)	Consales et al., 1990 (*40*)
Human neuroblastoma (IMR-32) cells	Conti et al., 1990 (*104*)
Chinese hamster ovary (CHO) cells	Burger et al., 1991 (*105*)
Lymphocytes	
Mouse macrophages (p388D1)	King et al., 1984 (*86*)

virus from WI-38 cells was only about one-tenth of that produced when BHK-21 and Vero cell lines were used.

The quality of the cells at the time of initial infection is critical and in order to obtain the maximum yield of virus from monolayer cultures, it is essential to use those that are just confluent, having been passaged not more than 2–3 days previously (*31*). Alternatively, high yields of virus can be obtained when cells in suspension are infected and then allowed to form monolayers. High input multiplicities of infection (MOI) may occasionally lead to autointerference (*32–35*), although this is not normally observed. Nevertheless, efforts to produce single growth curves in rabies-infected cells have not been particularly successful, possibly because virus replication proceeds relatively slowly and because of the difficulty of producing synchronous cell populations (*29, 30*).

Cytopathology

In general, no clearly definable cytopathological effect accompanies the production of rabies virus in cell cultures. In infected BHK-21 cell monolayers, for example, the cells merely begin to "age" and detach more quickly from the supporting surface than uninfected control cells (*31*). In monolayers of chick embryo fibroblasts, however, the viability of infected cells is sufficiently affected to permit plaque formation (*32, 33, 35*).

Similarly, plaques were formed in a subline (S13) of BHK-21 cells adapted to growth in agarose suspension (*34*), and plaque-forming viruses were recovered from persistently infected BHK-21 cells (*36*). This led to the development of the first reliable plaque assay system for rabies virus. Plaque formation was also induced in Vero cells infected with rabies virus and rabies-related (Lagos bat and Mokola) viruses (*37*), and a reproducible plaque assay system for rabies viruses in CER cells was described (*38*).

In murine neuroblastoma cells infected with fixed rabies virus and rabies-related (Lagos bat, Mokola and Duvenhage) viruses, the cytopathological effects are frequently more severe than in BHK-21 cells. Duvenhage virus induced the formation of syncytia (*39*), and kotonkan and Obodhiang viruses, after adaptation, could be titrated by plaque assay.

In McCoy cells, both fixed and street rabies viruses caused cytopathological changes from 24 to 72 hours after infection, dependent upon MOI (*40*). The cytopathological effect was easily recognized and resembled that induced by vesicular stomatitis virus (VSV) infection. Higher titres of the PV virus strain were found in supernatants of McCoy cells than in those of Vero cells.

Persistent infection

Although rabies virus-infected cells can be maintained in culture for extensive periods of time without noticeable cytopathological effect, persistent infection may give rise to different effects, depending upon the cell system used. For example, endosymbiotic infection, as found in rabbit endothelial cells infected with the CVS strain, was characterized by the accumulation of only small intracytoplasmic inclusions in the cells, with no apparent interference with the mechanism of cellular replication throughout a period of more than 2 years (*41*). On the other hand, in

persistent infections of Nil-2 cells, the production of new virus was observed to reach its peak in 3–4 days and then to fall off sharply during the next 6–7 cell transfers (*42*, *22*). Fluorescent intracytoplasmic inclusion bodies remained present in all the cultured cells after the decline in virus production. The cultures became resistant to superinfection with VSV and an inhibitor with interferon-like properties was recovered from the culture medium.

After this phase of infection, there was a marked decrease in the number of antigen-containing cells and in the production of infectious virus, followed again by a gradual increase in the proportion of infected cells and in the production of infectious virus. These cycles of high and low levels of rabies infection and corresponding resistance to challenge with VSV recurred periodically (*26*).

A somewhat different outcome of persistent infection has been described in NA-C1300 cells infected with a rabies virus of skunk origin (*43*). Infected cells were subcultured every 3–4 days and the supernatants were monitored for the presence of infectious virus. Eventually, no infectious virus could be detected in the supernatant by cell culture or animal inoculation tests, even though 95–100% of the infected cells remained positive for viral nucleocapsid antigen. A similar study using persistently infected BHK-21 C13 cells, which were passaged 100 times without re-infection, found that infectious virus continued to be released into the supernatant (*44*).

Following the discovery by von Magnus that serial undiluted passage of influenza virus produced incomplete virus particles that interfered with the growth of infectious virus (*45*), a similar phenomenon was identified in cell cultures infected with rabies LEP Flury virus (*46*). These "defective interfering" (DI) particles have since been described in many rabies cell systems and with many virus strains, suggesting that their replication is influenced by host-cell factors and the virus strain (*47–49*).

The particles are between one-third and two-thirds as long as infectious particles and require the presence of homologous infectious particles to undergo replication (*49*). They are readily produced: for example, detectable levels of DI particles could be generated in the first undiluted passage of a cloned pool of HEP Flury virus (*50*). They are probably involved in the establishment of persistent *in vitro* infections (*49*, *51*).

Although long-term persistent infections with rabies virus in cell culture have been studied in considerable detail, so far there is not a clear understanding of the effects of the evolutionary changes found in the viral RNA genome and proteins, the role of DI particles, the effect of temperature-sensitive mutants or the effect of changes in the synthesis of virus-specific RNA and proteins on the course of rabies infections in nature (*52*).

Virus in infected cells

Although inclusion bodies associated with rabies infection of the brain cells were first described in 1903, it was not until much later that these "Negri bodies" were shown to contain a viral antigen (*53*) and viral particles were demonstrated within the inclusion bodies (*54*, *55*). In a study using ferritin-labelled antiserum, it was shown that similar inclusion bodies in BHK-21 cells, although containing virus particles, were largely composed of ribonucleoprotein (*56*).

Conventional histological staining techniques for rabies (see Chapters 4 and 5) have now been largely replaced by the fluorescent antibody (FA) technique which stemmed from the work of Coons & Kaplan (*57*) and was later adapted for the diagnosis of rabies (*53*) (see also Chapter 7). The FA technique has also become the method of choice for following the progress of infection in cell culture. With fixed cells, the antigen predominantly stained is the nucleoprotein (N protein) of the nucleocapsid, whereas staining of unfixed cells reveals mainly viral glycoprotein (G protein) located on the cell plasma membrane (*30*). Although the FA test is a reliable and sensitive technique for demonstrating the presence of rabies virus in cells, it does not necessarily provide a measure of the amount of infectious virus within the cells or of their potential to release virus into the culture medium.

The introduction of monoclonal antibodies (MAbs) to the rabies field (*58*) (see also Chapters 11 and 12) has provided specific probes for the determination of antigenic variation among rabies and rabies-related viruses. These antibodies are directed mainly against either the N protein (MAb-Ns) and are used on fixed infected cell cultures, or the G protein (MAb-Gs), for use in mouse neutralization tests. When employed for the laboratory analysis of isolates in an integrated system of rabies case surveillance, such probes can provide a great deal of information about the distribution, prevalence and transmission of antigenic variants, both within and between animal species (*59*).

Application of cell-culture methods

Diagnosis

A diagnostic capability that includes virus isolation and identification in cell cultures is highly desirable, even in countries where rabies is not endemic. Following numerous reports of the isolation of fixed and street viruses in BHK-21 cells, the finding in 1975 that these cells could be successfully used for both post-mortem and ante-mortem diagnoses and that results could be obtained far more quickly than with the mouse inoculation test was a fundamental step forward (*60*); in 1980 the use of these cells for routine diagnosis was proposed (*61*).

BHK-21 cells are now used in many diagnostic laboratories. They are hardy, require a relatively simple medium supplemented with inexpensive bovine serum, and since they do not require additional carbon dioxide to support growth, they are safer than open culture systems. By regular (3–4 day) transfer of stock bottles at a split ratio of 1:4, cells remain healthy and an 80-cm^2 bottle at 3 days usually contains sufficient cells to give 100 ml of cell suspension. Culture bottles surplus to immediate requirements can be stored at room temperature or at 4 °C and passage levels can be kept low by returning to these cells up to 3 weeks later, when after one or two passages they regain their rapid growth characteristics.

The method used at Weybridge over many years for isolating and identifying the rabies virus was established by an experiment in which a pool of BHK-21 cells treated with diethylaminoethyl-dextran (DEAE-dextran) was infected with the supernatant fluid from a 10% suspension of infected brain which had been vigorously shaken (not centrifuged) in a leakproof bottle and left for a few minutes to allow coarse particles to settle.

The mixture was divided between six 25-cm^2 bottles and 12 Leighton tubes containing coverslips (used to monitor the adaptation process), which were then incubated at 35 °C. Each bottle was used as a series of passages at 1, 2, 3, 4, 5 or 6 days, the cells in the bottles were trypsinized and a portion (25%) was used to infect a further bottle and two Leighton tubes. The most rapid adaptation took place when infected cells were passaged either daily or every 2 days.

Following the adaptation of several hundred virus strains from many different species and geographical locations, some general observations can be made:

— at the first passage, many "Negri-body"-sized and smaller inclusions are observed in the cells;
— at the second passage, some cells contain large amorphous masses of nucleocapsid antigen (presumed to be within infected cells transferred from the first culture), while others appear to be "newly infected";
— at the third passage, a similar picture is observed, except that at least 50% of the cells are infected. The addition of an excess of uninfected cells at this stage allows the preparation of microtitration plates for analysis using MAbs and of virus stocks. These stocks are frequently obtained in less time than is taken by the same inoculum to kill mice following intracerebral inoculation of street virus. Not all viruses adapt at the same rate, however, therefore passaging is continued until at least 50% of the cells are infected.

The potential of CER and mouse neuroblastoma cells for use in the diagnosis of rabies infection has also been evaluated (*15*, *62*). Murine neuroblastoma cells have been shown to be more susceptible to rabies virus infection than any other cell lines tested, and in some laboratories, virus isolation in cell culture (with neuroblastoma cells) has replaced intracerebral inoculation of mice for diagnosis (*63*, *64*) (see also Chapter 8).

Rabies virus assay

As already mentioned, a number of plaque assays have been developed for the quantification and cloning of rabies virus. The most widely used method is based on agarose-suspended BHK-21 S13 cells in which most culture-adapted fixed strains produce plaques after 5–7 days of incubation (*26*, *34*). The plaques obtained in chick embryo fibroblast (CEF) cell cultures were regarded more as proliferative foci than the type of dead cells of which plaques are usually composed (*32*, *33*). Other methods of plaque assay include BHK-21 cell monolayers with a cross-linked dextran overlay (*65*) and pig kidney cell monolayers with a carboxymethylcellulose overlay (*66*).

Plaque assays, however, do not always give consistent results, even when well-adapted fixed rabies virus strains are used. For virus titration, they have been largely superseded by fluorescent focus assays in which cells are incubated with serial dilutions of virus, then fixed and treated with fluorescent serum or MAb-Ns 1–4 days later. In these tests, titration end-points compare well with those obtained by intracerebral inoculation of mice (*62*). Similarly, inhibition of fluorescent focus formation can be used to measure virus-neutralizing antibody levels in serum (see Chapter 15). This method has been extended to antibody-binding tests for determining the potency of rabies vaccines (*67*) (see also Chapter 43).

Infected cell-culture fluids also include non-infectious virus particles and other virion components. The complement fixation test can be used to measure total virus antigen and the haemagglutination test using goose erythrocytes can be used to measure intact viral particles. However, the latter test requires a concentration of at least 10^6 plaque-forming units (PFU)/ml and can be carried out only in serum-free medium (*68*).

Pathogenesis

Cell culture of rabies viruses can be used to study the processes of infection, transcription, translation, replication, assembly and release. In addition, many of the effects of the virus on cellular metabolism can now be examined at the molecular level (see Chapter 3).

Most of the earlier studies of rabies infection in cell culture employed thin-section electron microscopy to visualize how infection occurs, the fate of the virus once it has entered the cells, and the processes of morphogenesis and release of the virus. However, the majority of these studies were made in BHK-21 or CER cells, despite the fact that in the infected animal virus replication occurs predominantly in nervous tissue. Studies of persistently infected NA-C1300 cells examined by scanning and freeze-fracture electron microscopy may provide a more accurate picture of what happens in natural infection (*69*).

The development of plaque techniques has allowed the selection of virus "clones" and mutants. The availability of suitable virus-neutralizing MAb-Gs has made it possible to select virus variants (*70*, *71*) with altered pathogenic (*70*) or protective potential (*72*) whose antigenic changes can be subsequently mapped. A comparison of the cell-to-cell spread of pathogenic parenteral virus and non-pathogenic variants *in vivo* (in the brains of infected adult mice) and *in vitro* (in BHK-21 and neuroblastoma cells) demonstrated that the differences in the pathogenicity of the two types of virus were correlated in both *in vivo* and *in vitro* systems (*73*). The differences between pathogenic and non-pathogenic virus infections were observed only in the neuroblastoma cells which, as mentioned above, share a number of characteristics with neurons and which may therefore serve as a more appropriate model for *in vitro* pathogenicity studies.

Despite intensive research, a specific cell receptor site for rabies virus has not yet been identified, although muscle spindles and motor end-plates in striated muscle have been shown to be involved (*74*). The role of the acetylcholine receptor (AChR) has also been the subject of much debate, following the observation that antigen could be detected by the FA test at neuromuscular junctions — an area rich in AChRs — shortly after rabies virus was added to mouse diaphragm muscle (*75*). However, tests in a number of cell-culture systems, some of which lack high-density AChRs, have indicated that this receptor is not necessary for rabies virus infection and that the susceptibility of different cell types does not depend on a single specific type of receptor (*76*, *77*).

A highly sophisticated compartmentalized technique has been developed for the culture of dorsal root ganglion cells. This method allows the infection and manipulation of neuronal extensions without exposing the neuronal soma to the rabies virus (*78*). The high binding affinity of the virus to unmyelinated neurites and its transfer by the neurites to the neural soma supports the view that, *in vivo*,

sensory nerves may be involved in the centripetal transfer of virus to the central nervous system.

Other determinants, however, may be involved in the attachment of rabies virus to cell surfaces. Studies using CER cells suggest that phospholipids (*79*) and carbohydrate moieties (*80*, *81*) may play a role in the early interaction between the cell membrane and virus. It has also been postulated that sialylated gangliosides (*82*) and sialic acid (*83*) may be involved.

The possibility of an immunological involvement in at least some cases of rabies virus infection, such as the "early death" phenomenon, has been explored (*84*). In a test using the P388DI mouse macrophage cell line (*85*), it was shown that rabies virus antiserum diluted beyond its neutralization end-point enhanced the ability of the virus to infect these cells (*86*).

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